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CATALYTIC ENZYME-BASED METHODS FOR WATER TREATMENT AND WATER DISTRIBUTION SYSTEM DECONTAMINATION

1. LITERATURE SURVEY

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RESEARCH AND TECHNOLOGY DIRECTORATE

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PREFACE

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CATALYTIC ENZYME-BASED METHODS FOR WATER TREATMENT AND WATER DISTRIBUTION SYSTEM DECONTAMINATION

1. LITERATURE SURVEY

1. INTRODUCTION

Drinking water distribution systems supplying large population centers must be considered as serious potential targets for terrorists. Contamination of distribution system equipment would result from adherence of contaminants to biofilms, tubercles and other corrosion products lining the pipes, or from permeation of the pipe material itself. Because of their non-toxic, non-corrosive, and environmentally benign properties, enzymes may provide an ideal method for the treatment of agents, pesticides or other chemical contaminants in drinking water systems, as well as the decontamination of pipes and other equipment with contaminant residue. Additionally, enzymes have been demonstrated to function in foams, sprays, lotions, detergents, and other vehicles that can be used in flowing water or on material surfaces.

Many special requirements need to be considerations in the application of enzymes to contaminated drinking water systems. Because of the large volumes of water contained in water distribution and treatment systems, a decontaminant will need to be active for a much longer time than in military operations. Since drinking water flows very quickly in pipes, methods are needed to ensure that the enzymes maintain sufficient contact with the contaminated water or materials.

The goal of this project is to identify, develop, and evaluate at least one enzyme-based method for treating flowing contaminated water, and one enzyme-based method for decontaminating drinking water pipes. A thorough literature search was undertaken to fully identify the potential of enzymes to treat contaminated drinking water and/or to decontaminate distribution systems equipment. The literature search considered the potential application of enzymes to a large range of possible water contamination scenarios, from groundwater to drinking water, from toxic industrial chemicals to chemical warfare agents.

2. POTENTIAL TARGETS AND ENZYME SYSTEMS

2.1 Organophosphorus Agents and Pesticides.

The chemical warfare (CW) agents that have gotten the most attention over the past six decades are the organophosphorus (OP) nerve agents. These materials were derived primarily from work conducted in the late 1920's and early 1930's and aimed at developing pesticides that were more efficient. Tabun was actually first described in the literature in 1902, but its toxicity was not known for several decades. These materials owe their toxicity to the inhibition of acetylcholinesterase, an

enzyme necessary for efficient functioning of neuro-neuro and neuro-muscular junctions. The CW nerve agents are commonly divided into two groups. The G-type agents (ex. sarin, soman) are primarily O-alkyl alkylphosphonates that generally have a fluoride-leaving group (phosphorofluoridates), with the exception of tabun in which it is cyanide. The structures of the classical G-type nerve agents as well as diisopropylfluorophosphate (DFP, a commonly used simulant) are shown in Figure 1. A recent review provides a detailed discussion about the chemistry and toxicity of the phosphorofluoridates as well as an overview on enzymes for their detoxification.¹

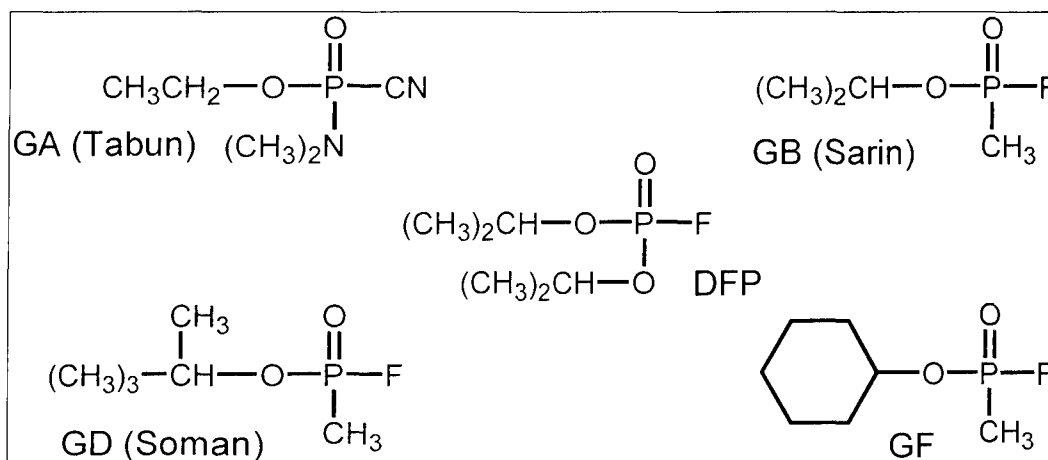


Figure 1. Structure of G-Type Nerve Agents and the Simulant DFP.

The V-type nerve agents are O-alkyl alkylphosphonothioates. The two primary ones are VX, developed and produced by the U.S. and its allies, and Russian VX (RVX or VR). Figure 2 shows the structures of these agents and several variants.

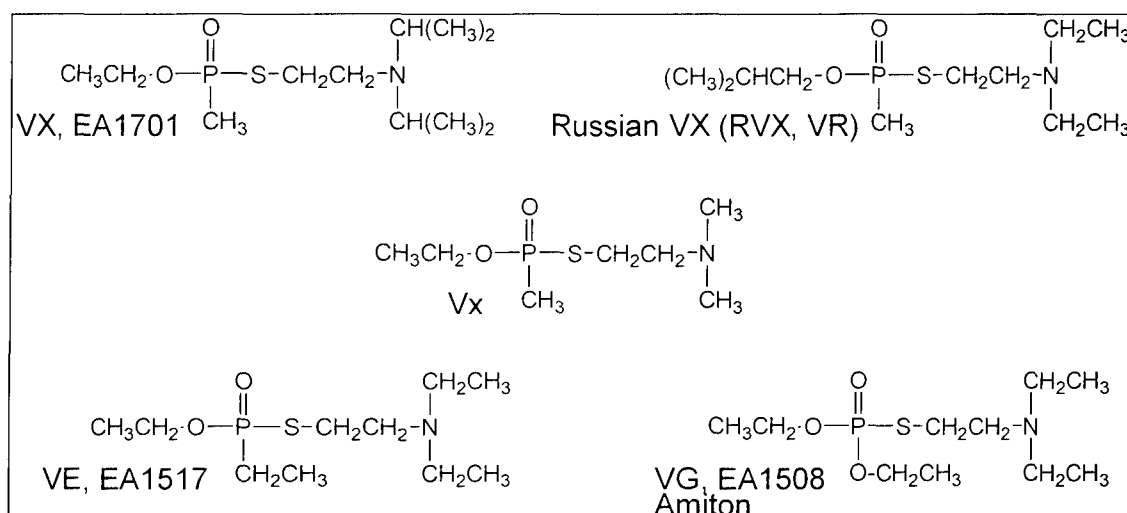


Figure 2: Structure of V-Type Nerve Agents.

In general, the V-agents are about an order of magnitude more toxic than the G-agents. They are also more stable with their hydrolysis rates several orders of

magnitude lower than for G-agents (at neutral pH). Unlike G-agents, V-agents can be detoxified by oxidation. Oxidation first attacks the nitrogen atom before proceeding to the sulfur atom and eventual cleavage of the P-S bond. However, in order to render the agents non-toxic, the oxidation (which requires 9 equivalents of oxidant) must go to completion since some of the intermediate products are still highly toxic. Since they have very low vapor pressures, the V-agents are considered percutaneous hazards, rather than by inhalation (with the exception of aerosols). As would be expected, there are varying degrees of similarity between the CW nerve agents and the pesticides they are derived from. A number of more common OP pesticides are shown below.

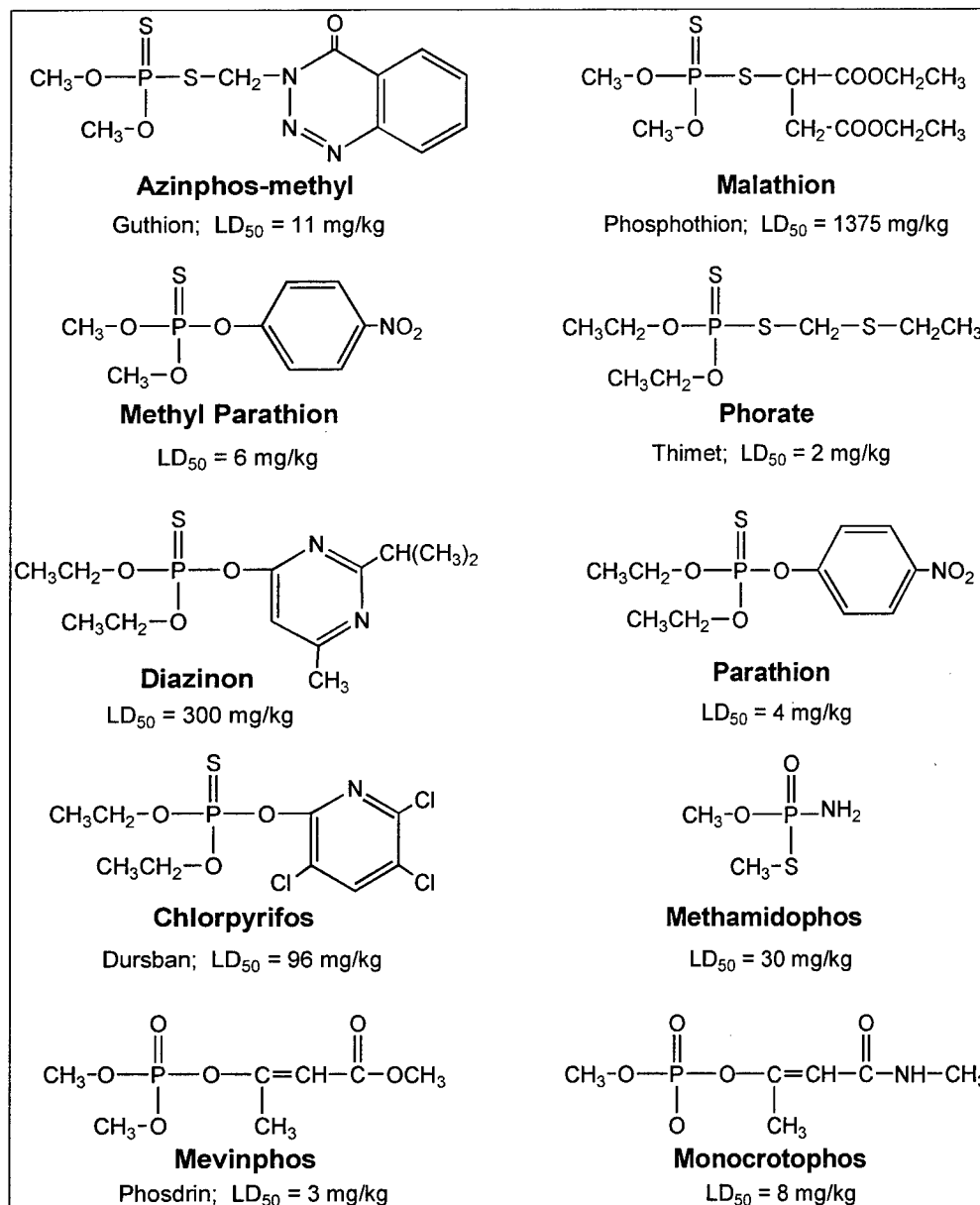


Figure 3. Common Organophosphorus Pesticides.

In general, the pesticides are phosphates or phosphothioates and as a result are lower in toxicity. Table 1 gives a comparison of the toxicity of the agents, some pesticides, and other toxic materials.

Table 1. Toxicities of Pesticides, CW Agents and Toxins.

Compound	Approx. LD ₅₀ (mg/kg, i.v.)
Diazinon	150-600
Coumaphos	90-110
EPN	35-45
Methyl Parathion	14
Parathion	13
Fensulfothion	5-10
Paraoxon	0.5
DFP	0.3
Sarin	0.01
Soman	0.01
Tabun	0.01
VX	0.001
Ricin	0.003
Palytoxin	0.00015
Botulinum toxin	0.000001

The ability of enzymes to hydrolyze and detoxify organophosphorus compounds goes back nearly 60 years to when Mazur published work with mammalian tissues conducted during World War II.² He determined that enzymatic activity in a variety of tissues could catalytically detoxify DFP.

During the 1950's three investigators, Aldridge³, Augustinsson⁴⁻¹⁰, and Mounter¹¹⁻¹⁶ carried out much of the work in this field. Aldridge reported on what he designated an A-serum esterase from mammalian sources that could hydrolyze paraoxon. Paraoxon is a product of mammalian oxidation of the pesticide parathion, but is considerably more toxic. This enzyme, more recently referred to as a phosphotriesterase or paraoxonase, was shown to differ from the phosphatases in that phosphatases only hydrolyze monoesters of orthophosphoric acid. In addition, he demonstrated that his A-serum esterases could be stereospecific, hydrolyzing (+)-sarin, but not the more toxic (-)-sarin.

Augustinsson confirmed the findings of Aldridge and extended the work to include enzymes that would hydrolyze organophosphorus compounds such as tabun. He determined that this phosphorylphosphatase or tabunase cleaved the P-CN bond of tabun to release hydrocyanic acid. Using a combination of electrophoretic separation,

substrate specificities, and sensitivity to inhibition, he concluded that there were three types of esterase activity in plasma: arylesterase (aromatic esterase, A-esterase), aliesterase (carboxylesterase, B-esterase, "lipase") and cholinesterase. He noted that there were species variations with respect to the properties of individual enzymes. He also confirmed the observation made by Aldridge that the hog kidney phosphorylphosphatases showed stereoselectivity against tabun.

During the same period, Mounter was attempting to further purify and characterize the enzyme from rabbit kidney originally reported by Mazur. He referred to this enzyme as dialkylfluorophosphatase (DFPase, fluorophosphatase). After partial purification by ethanol fractionation, the enzyme was determined to be activated by Co^{2+} and Mn^{2+} ions. Reagents that reacted with metal ions, sulfhydryl or carbonyl groups were found to inhibit DFPase activity. In addition to his work with mammalian enzymes, Mounter was the first to report on the DFPases in microorganisms. Of the bacteria tested, the highest activity was observed with the Gram-negative *Proteus vulgaris* and *Pseudomonas aeruginosa*, which were stimulated by Mn^{2+} . Based upon enzyme activity with different metal ions and inhibitors, he also demonstrated that there were a number of different DFPases. Studies conducted with preparations from *Escherichia coli*, *Pseudomonas fluorescens*, *Streptococcus faecalis*, and *Propionibacterium pentosaceum* showed that while there were differences in the relative hydrolysis rates of a variety of organophosphorus compounds, they were comparable to those observed with the hog kidney enzyme.

During the late 1950's and early 1960's, a number of additional groups became involved in the investigation of the enzymatic hydrolysis of DFP, paraoxon, sarin and tabun.¹⁷⁻²⁴ While considerable advances were made during this time in comprehending the diversity of enzymes, one of the most significant events was the beginning of the research efforts of Prof. Francis Hoskin in this field. Of particular importance is his work, beginning in 1966, in the purification and characterization of a DFPase from squid.²⁵⁻²⁷ The squid (*Loligo pealei*) enzyme has a molecular weight of approximately 30 kDa, is found only in cephalopods, requires Ca^{2+} for activity, and hydrolyzes DFP five times faster than soman. The significance of the squid enzyme lies in the fact that its chemical and biological properties are completely different from all other types of DFPases. To this day, it still appears to belong to a unique class of enzymes. The gene for a squid (*Loligo vulgaris*) enzyme was cloned, sequenced, expressed in *E. coli* and in the yeast *Pichia pastoris*, and further characterized by Prof. Heinz Rüterjans, University of Frankfurt, Germany.²⁸⁻³⁰ The recombinant enzyme had a molecular weight of 35.2-kDa. Earlier work by Rüterjans, Hoskin and others indicating a lower molecular weight was probably due to proteolysis during purification. The enzyme was crystallized and its three-dimensional structure determined.^{31,32} It was determined that one of the Ca^{2+} ions was required for stabilizing the enzymes structure, whereas the second was likely to fulfill a catalytic function. Roche Diagnostics, Berne, Switzerland, conducted an initial industrial scale production run (15 kilograms) of the recombinant DFPase. However, Roche has decided not to continue with this effort. Prof. Rüterjans, the holder of a patent on the enzyme,³³ is currently in the process of obtaining a new commercial production source.

The interest in microbial enzymes for the degradation of organophosphorus compounds received a boost in the early 1970's by the isolation of bacteria capable of growing on a variety of pesticides. The initial report was by Sethunathan and Yoshida who isolated a diazinon-degrading *Flavobacterium* species (ATCC 27551) from rice paddy soil.³⁴ Cell-free extracts of this organism could also hydrolyze the insecticides chlorpyrifos (diethyl (3,5,6-trichloropyridyl) phosphorothionate) and parathion, the aromatic or heterocyclic products of which were not further metabolized. In 1973, a pseudomonad capable of hydrolyzing parathion and utilizing the *p*-nitrophenol product as a source of carbon and nitrogen was isolated.³⁵ Somewhat later, Daughton and Hsieh isolated a *Pseudomonas stutzeri* capable of hydrolyzing parathion from a chemostat culture.³⁶ They also described strains of *Bacillus* and *Arthrobacter* that can hydrolyze parathion³⁷ as well as a *Pseudomonas* capable of utilizing isofenphos as sole carbon and energy source.³⁸ In 1979, Rosenberg and Alexander³⁹ described two *Pseudomonas* isolates capable of hydrolyzing a variety of organophosphorus compounds and using the products as sole phosphorus source. With the exception of the one from *Flavobacterium*, little if any additional information is known about any of these organisms or their enzymes.

In addition to the *Flavobacterium* mentioned above, the other major parathion-degrading bacterium described in the literature is *Pseudomonas diminuta* MG, which was isolated in 1976 by Munnecke.^{40,41} By far, these two enzymes have been the most widely studied of any capable of hydrolyzing organophosphorus compounds. However, it is worthwhile to note that while the two organisms containing the enzymes were isolated on opposite sides of the world and the plasmids that contain their genes are highly dissimilar, the genes (and enzymes) themselves are virtually identical.⁴² These phosphotriesterases, parathion hydrolases or organophosphorus hydrolases are also the only well-characterized enzymes known to catalytically hydrolyze the P-S bond of V-agents.^{43,44} This enzyme will be discussed in much more detail in a later section.

Beginning in the 1980's, the research efforts in this field have been divided into two major areas: the isolation and characterization of microorganisms (and their enzymes) capable of growth on a variety of organophosphorus pesticides; and the somewhat more random search for organisms that possess enzymes capable of hydrolyzing DFP and the related nerve agents. The relatively high spontaneous hydrolysis rates of the nerve agents make their use as substrates in microbial enrichment cultures rather problematic. Generally, microorganisms that grow on them actually utilize the hydrolysis products of the agents and do not possess the enzymes to deal with the agents themselves. Examples of the former are the isolation of *Pseudomonas alcaligenes* C1 that can hydrolyze and grow on fensulfothion;⁴⁵ the isolation of additional *Pseudomonas* sp.; and other unidentified bacteria that hydrolyze and grow on parathion and/or methyl parathion;^{46,47} and the isolation of three distinct bacteria capable of metabolizing coumaphos.⁴⁸

In the search for nerve agent degrading enzymes, investigations that are more recent have gone in a number of directions. Landis and co-workers examined the

ciliate protozoan *Tetrahymena thermophila*⁴⁹⁻⁵¹ and the clam *Rangia cuneata*.⁵² Partial purification of extracts from *Tetrahymena* revealed that this organism had at least five enzymes with DFPase activity and molecular weights ranging from 67-96 kDa. The rate ratios for soman and DFP hydrolysis as well as the effect of Mn^{2+} on activity varied considerably from one enzyme to another. Preliminary investigations on the clam also resulted in the detection of several DFPases with differing substrate specificity and metal stimulation. Of particular interest was the presence of an enzyme in the clam digestive gland that appeared to have significant activity on the DFP analog mipafox. Most enzymes described to date are either indifferent to mipafox or subject to strong competitive inhibition.

Little et al. characterized an enzyme from rat liver that has a substrate preference of sarin > soman > tabun > DFP, but without activity on paraoxon.⁵³ The enzyme had a molecular weight of 40 kDa and was stimulated by Mg^{2+} . Unlike an enzyme from *Escherichia coli*, all the stereoisomers of soman appeared to be hydrolyzed at equal rates.

A screen of 18 Gram-negative bacterial isolates by Attaway et al. resulted in the finding that, while all showed at least some activity on DFP, only cultures with parathion hydrolase activity showed significant "DFPase" levels.⁵⁴

In the mid 1980's researchers at ECBC began an investigation of thermophilic bacteria as sources of DFPases. A Gram-positive, aerobic, spore-forming, rod-shaped, obligate thermophile was isolated from the soil of Aberdeen Proving Ground and found to possess activity against DFP and soman.⁵⁵ This isolate, designated as JD-100, was tentatively identified as a strain of *Bacillus stearothermophilus*. Its temperature range for growth was from 40-70°C, with an optimum of ~55°C. While crude cell extracts of JD-100 had low levels of DFP activity, the purified enzyme showed no detectable DFP activity but retained soman hydrolyzing activity.^{56,57} The enzyme had a molecular weight of 82-84 kDa and showed considerable stimulation by Mn^{2+} (~80-fold). It was unaffected by mipafox and appeared to degrade all the stereoisomers of soman. It should be noted that at high (>3 mM) concentrations of GB, GD, and GF, the fluoride ion that is released catalyzes the racemization of the stereoisomers^{58,59} of these compounds so that even if only one is being degraded, the other will be converted so that hydrolysis will go to completion.⁶⁰

As illustrated in the discussion above, the nomenclature of these enzymes has been unsystematic and confusing. In general, the names utilized have been representative of the particular substrate used by an individual investigator. Hence, the literature is filled with references to enzymes such as phosphorylphosphatase, fluorophosphatase, DFPase, paraoxonase, parathion hydrolase, phosphotriesterase, phosphofluorase, somanase, sarinase, and tabunase. In 1992, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology developed a new nomenclature for use until the natural substrates and functions of these enzymes could be determined. Under the general category of Phosphoric Triester Hydrolases (EC 3.1.8), the enzymes are divided into two subgroups. The first, EC 3.1.8.1,

Organophosphate Hydrolase (also paraoxonase and phosphotriesterase) is for those enzymes with paraoxon and other P-esters (P–O bonds) as preferred substrates. The second group, EC 3.1.8.2, Diisopropyl-Fluorophosphatase (also Organophosphorus Acid Anhydrolase or OPAA) is for enzymes with a preference for OP compounds with P–F or P–CN bonds.

The Organophosphorus Hydrolase (OPH) (parathion hydrolase, or phosphotriesterase) from *P. diminuta* or *Flavobacterium* has been one of the most studied enzymes in regards to its activity on nerve agents and pesticides. While many researchers have studied OPH, the primary information on the structure and function of the enzyme has come from the laboratories of Rauschel and Wild, both at Texas A&M University. Most early work with the enzyme used the constitutively expressed form which is membrane associated.⁶¹ When the gene for the enzyme was cloned into other hosts, the membrane association remained, making purification difficult. Serdar et al., discovered that the enzyme was synthesized as a 365 amino acid precursor from which 29 amino acids were removed to generate the mature protein.⁶² When this 29 amino acid leader sequence was removed from the clone, the recombinant enzyme was found as a soluble mature enzyme that maintained activity. The enzyme has been expressed in a variety of hosts including insects,⁶³ insect cells,⁶⁴ fungi,⁶⁵ and *Streptomyces*.^{66,67}

The mature enzyme is a ~ 36-kDa metalloprotein with two Zn^{2+} ions present in the native enzyme. However, one can substitute a variety of other divalent metal ions (Mn^{2+} , Cd^{2+} , Co^{2+} , or Ni^{2+}).⁶⁸ The Co^{2+} enzyme has the greatest activity on substrates with P–F and P–S bonds. Originally thought to function as a monomeric enzyme, more recent information, based on the crystal structure of the protein, indicates that the active form is actually a homodimer.⁶⁹

OPH has a broad pH profile with optimum activity between pH 8–10 and a temperature optimum of ~50°C. OPH has the ability to hydrolyze a wide variety of organophosphorus pesticides⁷⁰ as well as other compounds having P–O, P–F, and P–S bonds. Several selected substrates and the kinetic constants for OPH (Co^{2+} form) are shown in Table 2.

Table 2. Comparison of Hydrolytic Constants for OPH.

Substrate	Bond type	k_{cat} (sec ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ sec ⁻¹)
Paraoxon	P-O	3170	0.058	5.5×10^7
Parathion	P-O	630	0.24	2.6×10^6
DFP	P-F	465	0.048	9.7×10^6
Sarin	P-F	56	0.7	8.0×10^4
Soman	P-F	5	0.5	1.0×10^4
Demeton-S	P-S	1.25	0.78	1.6×10^3
VX	P-S	0.3	0.44	45
Acephate	P-S	2.8	160	18

Assays conducted at pH 7.2 and 37 °C

The hydrolytic mechanism of OPH was originally believed to involve an S_N2 mechanism where an active site base of the enzyme abstracts a proton from a water molecule, thus making it a nucleophile that directly attacks the phosphorus in the substrate.⁷¹ Based on the crystal structure of the enzyme it is now thought that the active site base is not required. The binuclear metal center of the enzyme is located at the C-terminus of a β -barrel with the metals separated by 3.8Å. There are two bridging ligands to the metals: a water molecule (or possibly a hydroxide ion) and a carbamylated lysine residue. The bridging water molecule probably acts as the nucleophile in the reaction with the substrate by single in-line displacement attack.⁷² Site-directed mutagenesis of OPH has resulted in modification of its activity on a variety of substrates.^{73,74} Both the catalytic activity (k_{cat}) and substrate specificity (k_{cat}/K_m) of OPH has been changed as well as its stereospecificity against chiral substrates. Increases in activity of 20-40 fold have been achieved with soman and to a lesser extent with DFP, VX and acephate. The natural or original function of OPH is still unknown. So far, this type of enzyme has been restricted to prokaryotic organisms. However, with fluorescent *in situ* hybridization (FISH), cDNAs encoding proteins with a high degree of homology to OPH have been identified in mice,⁷⁵ rats,⁷⁶ and humans.⁷⁷

OPH is the only well characterized enzyme with demonstrated catalytic activity against V-agents. However, recent reports by Liu et al. describe two fungal enzymes that need to be examined for this potential capability. These enzymes appear to be quite different from any others yet reported. One was obtained from a strain of *Aspergillus niger* isolated from dimethoate-contaminated sewage and soil.⁷⁸ This 67-kDa enzyme has activity against dimethoate, formothion and malathion, but none against parathion. The second enzyme, from *Penicillium* is similar in size, 60-kDa, but has a substrate specificity more similar to OPH.⁷⁹ It is able to hydrolyze methyl parathion, parathion, paraoxon, coumaphos, demeton-S, phosmet, and malathion. In addition to the size difference, these are also unusual in that they appear to be activated by Cu^{2+} . Nothing is yet known about their activity against organophosphorus nerve agents.

Work began in the late 1980's at ECBC on identifying OPAA enzymes in halophilic (salt loving) bacteria. The rationale behind this was the intent to develop enzyme-based decontamination systems that would use any available water, to include seawater and other saline sources. Bacterial isolates were obtained from water and soil samples of salt springs in the state of Utah. One particular isolate, designated JD6.5 was obtained from Grantsville Warm Springs, which has a relatively constant temperature of 24-32°C and 25,000 ppm dissolved solids (96% NaCl). The isolate is a Gram-negative, aerobic short rod, and an obligate, moderate halophile. It required at least 2% NaCl for growth, with an optimum between 5-10% NaCl. Fatty acid analysis of the isolate identified it as a strain of *Alteromonas*, a common genus of marine bacteria. An intracellular OPAA enzyme from strain JD6.5 was purified and characterized.⁸⁰ It is a single polypeptide with molecular weight of 58 kDa. It has a pH optimum of 8.5 and temperature optimum of 50 °C. Maximum activity is with either Mn²⁺ or Co²⁺. The enzyme is inhibited by iodoacetic acid, *p*-chloromercuribenzoate, and *N*-ethylmaleimide, indicating that a sulfhydryl group is either essential for activity or in close proximity to the active site. It is subject to competitive, reversible inhibition by mipafox and is significantly stimulated by NH₄⁺ ions (3-5 fold). Its catalytic activity against nerve agents and related compounds is shown in Table 3.

Table 3. OP Substrate Specificity for *Alteromonas* sp. JD6.5 OPAA.

Substrate*	k_{cat} (sec ⁻¹)
GD (soman)	3145
DFP	1820
GF	1654
GB (sarin)	611
Paraoxon	124
GA (tabun)	85
VX	0

* The activity on the substrates with fluoride leaving groups was measured with a fluoride ion-selective electrode method. Activity on paraoxon was determined by measurement of the increase in absorbance at 405 nm representing the release of the *p*-nitrophenol group. For GA (tabun), cleavage of the P-CN bond was determined by P³¹-NMR.

The activity with soman corresponds to a 10^9 -fold increase in the rate of reaction compared to its spontaneous hydrolysis rate. It was also one to two orders of magnitude greater than for any other known enzyme.

Having identified isolate JD6.5 as a strain of *Alteromonas*, a number of other *Alteromonas* strains were obtained from the laboratory of Dr. Rita Colwell, University of Maryland, and the American Type Culture Collection (ATCC). These strains were evaluated for enzyme activity against DFP the nerve agents.⁸¹ Several showed high levels of activity and two of the enzymes were purified and characterized.^{82,83} Table 4 shows how these enzymes compare to the JD6.5 strain OPAA in physical and biochemical properties. There are significant similarities as well as differences with these enzymes. In order to produce the enzymes in larger quantities, the genes for *A. sp.* JD6.5 and *A. haloplanktis* were cloned into *E. coli* and expressed.⁸⁴ In addition, the gene sequences were determined and translated into an amino acid sequence. The 10-kDa molecular weight difference between the *A. sp.* JD6.5 and *A. haloplanktis* OPAA's was found to be due to the presence of an extended C-terminal region in the JD6.5 enzyme. The two enzymes have a 77% amino acid homology. If the extended C-terminus of the JD6.5 enzyme is excluded, the homology increases to ~90%.

Previously, it had been assumed that the natural function of the OPAA's would have something to do with phosphorus metabolism (phospholipase, phosphodiesterase, etc.). Therefore, it came as a considerable surprise when the results of screening the amino acid sequence of *A. sp.* JD6.5 against the NCBI protein database revealed a high degree of homology (48%) to the *E. coli* X-Pro dipeptidase. Two other matches were for *E. coli* aminopeptidase P (31% homology) and *Lactobacillus sake* dipeptidase (19% homology). There was no homology observed between the OPAA and the *Flavobacterium* or *P. diminuta* phosphotriesterase OPH or squid DFPase.

Table 4. Comparison of *Alteromonas* OPAA's.

OPAA Property	<i>Alteromonas</i> sp. JD6.5	<i>Alteromonas</i> <i>haloplanktis</i>	<i>Alteromonas</i> <i>undina</i>
Molecular Weight (kDa)	60	50	53
pH Optimum	8.5	7.5	8.0
Temperature Opt. (°C)	50	40	55
Metal Requirement	Mn = Co	Mn	Mn
Substrate Specificity (k_{cat})			
DFP	1820	691	1403
GA	85	255	368
GB	611	308	426
GD	3145	1667	2826
GF	1654	323	1775

X-Pro dipeptidases, also known as Prolidases (EC 3.4.13.9), are a ubiquitous class of enzymes that hydrolyze dipeptides with a prolyl residue at the carboxyl-terminal position. They are usually activated by Mn^{2+} , are possibly thiol dependent, and usually do not act on tri- or tetrapeptides or dipeptides with proline at the N-terminus. They generally have a molecular weight of 40-50 kDa, a temperature optimum between 40 and 55 °C, and a pH optimum between 6.5 and 8.0. All these properties are very similar to those of the *Alteromonas* OPAA's.

Table 5. *Alteromonas* OPAA Dipeptidase Profile.

Substrate	Specific Activity (μ moles/min/mg protein)		
	<i>Alteromonas</i> sp. JD6.5	<i>Alteromonas</i> <i>haloplanktis</i>	<i>Alteromonas</i> <i>undina</i>
Leu-Pro	636	988	810
Ala-Pro	510	725	658
Leu-Ala	82	63	220
Gly-Glu	<1	<1	1391
Met-Asn	<1	<1	410
Ala-Ala	<1	<1	105
Pro-Leu	<1	<1	<1
Pro-Gly	<1	<1	<1
Gly-Pro-Ala	<1	<1	<1
Ala-Pro-Phe	<1	<1	<1

The three OPAA's listed in Table 4 were then tested against a variety of di- and tripeptides by measuring the release of amino acids by a modified Cd-ninhydrin method.⁸⁵ The results of these assays are shown in Table 5. The results clearly indicate that the *Alteromonas* OPAA's are prolidases rather than aminopeptidases.

Hoskin and Walker examined several nerve agent/DFP-hydrolyzing enzymes to determine whether any of these might also be prolidases.⁸⁶ Rather than measuring dipeptide hydrolysis directly, they reasoned that if a DFP-hydrolyzing enzyme also hydrolyzes Leu-Pro, then Leu-Pro should inhibit the hydrolysis of DFP. Their results are shown in Table 6.

Table 6. Effect of Leu-Pro on DFP Hydrolysis by Purified Nerve Gas/DFP Hydrolyzing Enzymes in Relation to Other Properties.

Enzyme Source	% Inhibition by Leu-Pro	Soman/DFP Hydrolysis ratio	Mn ²⁺ Stimulation
<i>Loligo pealei</i> (squid)	-3, -5*	0.2-0.25	0
<i>Pseudomonas diminuta</i> OPH	0, 2, 3	0.125	0
Hog kidney	93, 93	~5	~5X
<i>Escherichia coli</i> OPAA	72, 76	~50	~5X
<i>Alteromonas</i> JD6.5 OPAA	55, 68	~2	Yes

*Negative values = stimulation

This result demonstrated that the hog kidney and the *E. coli* OPAA's most likely are prolidases and that the squid-type OPAA and the *P. diminuta* OPH are not. This does not eliminate the possibility that the squid-type enzyme may be a peptidase with different substrate specificity. However, for both OPH and the squid DFPase, the fact that they have no sequence homology suggests that they have very different natural functions than the prolidases.

Since the *Alteromonas* prolidases have high levels of activity against the G-type nerve agents, the question arises whether other prolidases have OPAA activity as well. In the case of mammalian enzymes, partially purified human and porcine liver prolidases were obtained from Dr. Lin Liu, ChemGen Corporation and Sigma Chemical Co. respectively. Both had low levels of activity against DFP and G-agents, in the range of 1/200-1/500th of that seen with the OPAA's. Their activity on X-Pro dipeptides was comparable to that observed with the OPAA's.

At the other extreme, a preparation of recombinant prolidase from the hyperthermophile *Pyrococcus furiosus* was obtained from Dr. Michael Adams, University of Georgia.⁸⁷ It was determined that it had measurable, but low levels of DFP activity at 80°C. The activity measurements were well below the optimum of this enzyme (100°C), but at the maximum temperature that the fluoride electrode could tolerate. For safety reasons, the high temperature tests were not repeated with the nerve agents. This enzyme is a homodimer with 39.4 kDa MW subunits and a pH optimum of 7.0. In regards to metal requirements, Co²⁺ is preferred 4:3 over Mn²⁺. The preferred substrates are Met-Pro and Leu-Pro. Comparison of the amino acid sequence of this enzyme with other prolidases found the greatest degree of similarity with that from another archeon, *Methanococcus jannaschii* (69%). Considerable similarity was also found with other prolidases for which the sequence is known: *Lactobacillus delbrueckii* (61%), *Haemophilus influenzae* (58%), *E. coli* (56%), human (53%), and *A. sp.* JD6.5 (51%). Based on the crystal structure of the *E. coli* methionine aminopeptidase, five amino acids (Asp⁹⁷, Asp¹⁰⁸, His¹⁷¹, Glu²⁰⁴, and Glu²³⁵) were shown to coordinate the binding of two Co²⁺ ions per active site.⁸⁸ All these prolidases

conserved the same five residues, even though some of the enzymes are monomers instead of dimers. In the case of strain JD6.5, the conserved residues are: Asp²⁴⁴, Asp²⁵⁵, His³³⁶, Glu³⁸¹, and Glu⁴²⁰.

The question naturally comes up as to why prolidases are such efficient catalysts for the hydrolysis of organophosphorus compounds, in particular, for the nerve agents. Molecular modeling studies comparing the structures of soman and Leu-Pro have been carried out.⁸⁹ It was determined that the three-dimensional structure and the electrostatic density maps of the materials look nearly identical. The organophosphorus compounds such as soman appear to fit into the active site of the enzyme in an orientation that allows the hydrolysis of the target P-F, P-CN, or P-O bond. Using the crystal structure of the *E. coli* methionine aminopeptidase, a postulated model of the *A. sp.* JD6.5 active site has been developed. It indicates two hydrophobic pockets, a large one where the side chain of the leucine (of Leu-Pro) can fit and a smaller one where the proline ring fits. The amide bond is positioned for a backside attack just above the two metal ions that have a bridging oxygen or hydroxyl group. When soman is substituted for Leu-Pro, the pinacolyl group fits into the large hydrophobic pocket and the methyl group into the small pocket. The phosphorus atom is located in the same position as the amide bond of Leu-Pro and the fluorine leaving-group extends out of the active site. The model suggests that the activity of the dipeptidases on soman and related compounds is primarily a matter of serendipity. They mimic the structure of the natural substrates for the enzyme so well that the enzymes are able to efficiently catalyze their hydrolysis. Confirmation of this proposition will be based on the crystal structure of the *A. sp.* JD6.5 enzyme. While Dr. Quoicho, Baylor University, has determined the structure of the enzyme, it has not yet been published.

As mentioned earlier, recombinant OPH can be produced in a variety of host organisms. However, in most cases the level of production has been relatively low 10-25 mg/L. In some instances, it is believed that the enzyme forms insoluble and inactive inclusion bodies, but this has not yet been confirmed. A variety of techniques such as varying growth temperature, differing the carbon sources in microbial fermentations, fed-batch fermentations, and others are being pursued. The use of a host that will secrete the active enzyme may also solve this problem, although production by *Streptomyces* was still quite low. As reported in a recent patent, slight modification of the recombinant gene has improved the yield considerably. In addition, an improved method of purification has resulted in a near one-step process.⁹⁰

The *A. sp.* JD6.5 prolidase has proven to be quite amenable to production by recombinant DNA technology. In initial studies, the *opaA* gene encoding the prolidase was cloned into pBluescript SK+ (pTC6513) and expressed in *E. coli*. The expressed enzyme constituted about 5% of the total cell protein. To further enhance production, the gene was cloned downstream of a strong *trc* promoter in a high-level, regulated expression vector, pSE420 (Invitrogen, San Diego, California). After induction with IPTG, the enzyme was produced at levels up of to 50-60% of total cell protein for a yield in shake-flask cultures of 150-200 mg/L.⁹¹ Fermentation studies with 10-liter fed-batch systems have pushed the levels to nearly 1 g/L. At ECBC, fermentation studies

were conducted up to the 1000-liter scale for both OPAA and OPH. Quite recently, it was determined that instead of the expensive IPTG, the natural inducer lactose would give comparable levels of production at a much-reduced cost.

In early 2004, the OPH patent and two patents on the *A. sp.* JD6.5 prolidase^{92,93} were licensed to Genencor International, Inc., for large-scale production of the enzymes. Genencor is the largest U.S. producer of industrial enzymes and second in the world only to Novozymes. In their initial trial fermentations (14 and 3000 liters), the levels of OPH and OPAA have exceeded the results obtained at ECBC.⁹⁴ The U.S. EPA has approved production of the enzymes and their commercial sale will begin in late summer of 2004. Genencor is currently scaling up the process to 30,000 liters.

In order to be useful in the decontamination of chemical or biological agents during military operations, after terrorist attacks, or accidental release, any enzyme-based formulation needs to be stable for long periods and easy to use. Both OPH and the *A. sp.* JD6.5 prolidase has been lyophilized in the presence of trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) and stored for extended periods (>5 years) at room temperature with no apparent loss of activity.⁹⁵ In the absence of trehalose, lyophilized enzyme lost >90% of its activity. While useful for small quantities of enzymes, it is too difficult and expensive for the production of industrial scale quantities. In addition to improved production levels, preliminary studies at Genencor have shown that both enzymes are amenable to granulation, which will be a much less expensive operation than freeze-drying. For example, enzymes can be found in this form in powdered laundry detergents.

Enzyme-based decontamination formulations are planned to be reconstituted in whatever water-based system the user has available. The systems being considered for dealing with contaminated vehicles, facilities and terrain include fire-fighting foams and sprays, aqueous degreasers, laundry detergent, aircraft deicing solutions, etc. Table 7 shows the effect of a variety of these types of materials on the activity of the prolidase. The materials were evaluated at the shown concentrations. OPH has been the workhorse for a number of groups in evaluating activity in unusual environments.

Both the prolidase and OPH have been shown to be active in generated foams.⁹⁶ The use of foams offers several advantages. The foams are generally made up of surface-active agents that may help in the solubilization of the substrates, and the foam will stick to vertical surfaces for sufficient time to allow the enzyme action. As can be seen in Table 7, there is considerable variation in activity, but even in the systems where inhibition occurs, the residual activity may be sufficient to carry out the necessary decontamination. It should be noted that the enzyme was not optimized for use in any of these materials and that considerable enhancement may be possible.

In addition to these liquid matrices, the prolidase and OPH were immobilized in polyurethane foams where they retained significant activity.⁹⁷ The immobilized enzymes were considerably more stable than the free enzyme. This offers

the potential use of the enzymes in sponges or wipes for the decontamination of personnel (including casualties) and small sensitive equipment. This is also relevant to one of the goals of this project, the development of enzyme filters.

The potential for enzymes to function on a large scale against nerve agents was demonstrated under the auspices of NATO Project Group 31 (PG.31) that deals with the development of "Non-Corrosive, Biotechnology-Based Decontaminants for Chemical and Biological Agents." PG.31 currently consists of the Czech Republic, France, Germany, Italy, Turkey, the United Kingdom, and the United States (lead nation). In addition, observers have participated from Belgium, Denmark, and Poland. Successful trials with soman and VX were conducted in France, Germany and the United Kingdom. These trials have used enzymes in a variety of matrices (foams, sprays and microemulsions) against agents on both porous and hardened painted surfaces. Excellent removal and hydrolysis of the agents was observed.

To summarize this section, it appears that all hydrolytic enzymes for the catalytic detoxification of organophosphorus nerve agents and pesticides are variations of three types: squid DFPase, OPH or prolidase (OPAA). There has been some work on the enzymatic oxidative decontamination of V-type nerve agents, but this is non-specific in nature and the enzymes will be discussed in section 2.3.

2.2 Carbamate Pesticides.

The natural carbamate of greatest significance is physostigmine. It is derived from the West African Calabar bean. The use of synthetic carbamates as insecticides began in the 1950's and as fungicides in the 1970's. In 1986, it was estimated that the worldwide use of carbamate pesticides was in the 20,000-35,000 ton range.⁹⁸ By 1995-6, the U.S. consumption of carbamate pesticides totaled nearly 19,000,000 tons (primarily consisting of aldicarb, carbaryl, carbofuran, and molinate).⁹⁹ In addition to carbamate pesticides, a number of carbamate herbicides were produced and used widely. Although their toxic effects are similar to the organophosphorus pesticides and they are used in large quantities worldwide, there has been much less work published on their biodegradation and metabolic pathways.

Table 7. DFP Hydrolysis by *Alteromonas* sp. JD6.5 Prolidase in Different Matrices.

Matrix (Source)	Normal Function	Conc. (%)	Spec. Act. (U/mg)*
Control (buffer only)	–	–	1950
AFC-380 (Sandia National Lab.)	modified fire-fighting foam	6	1050
AFFF (3M; St. Paul, MN)	fire-fighting foam	6	460
BioSolve (Westford; Westford, MA)	fire-fighting wetting agent	6	1030
ColdFire (FireFreeze; Rockaway, NJ)	fire-suppressing agent	10	2340
Silv-EX (Ansul; Marinette, WI)	fire-fighting foam	6	320
Blue Base (Neutron; Torrance, CA)	degreaser	8	140
BV406LF (FireFreeze; Rockaway, NJ)	degreaser/cleaner	10	1430
Green Thunder (Jackson, MI)	degreaser/cleaner	10	250
SC-1000 (Gemtek; Phoenix, AZ)	wetting agent/ degreaser	5	650
Star Clean Miracle (Hudsonville, MI)	wetting agent/oil remover	30	670
Supersolve (Gemtek; Phoenix, AZ)	wetting agent/ degreaser	10	440
Odor Seal (FireFreeze; Rockaway, NJ)	wetting agent/odor remover	10	1980

* The reaction medium contained 50 mM $(\text{NH}_4)_2\text{CO}_3$ (pH 8.7), 0.1 mM MnCl_2 , 3 mM DFP, and 0.3-0.4 units of enzyme in a total volume of 2.5 ml. One unit (U) of enzyme activity is defined as the release of 1.0 mmole of $\text{F}^- \text{ min}^{-1}$.

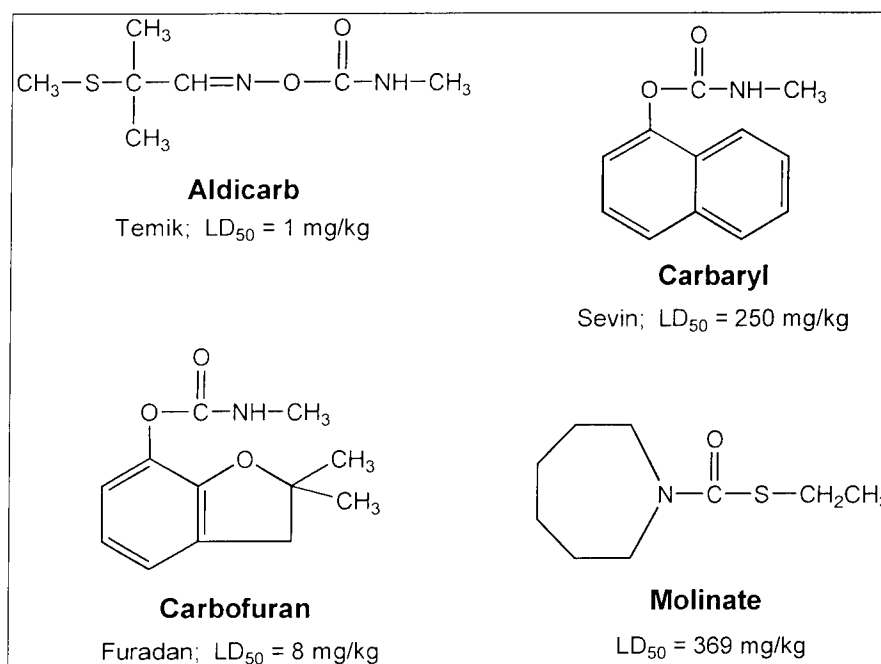


Figure 4. Common Carbamate Pesticides.

In 1965, Kearney published a paper on the purification of an enzyme capable of hydrolyzing phenylcarbamates,¹⁰⁰ but little else was done on carbamate enzymes for the next twenty years. In 1968, the metabolism of carbofuran (also known commercially as Furadan) in rats and houseflies was described.¹⁰¹ The first report on degradation of carbamate pesticides was in 1970.¹⁰² Getzin first reported on the degradation of carbofuran in soils in 1973.¹⁰³ Over the next 30 years, a number of groups described the biodegradation of carbamate pesticides (primarily carbofuran) and herbicides by a variety of microorganisms.¹⁰⁴⁻¹¹⁵ Early in this period, the proposed mechanism for the biodegradation and metabolism of carbofuran and carbaryl was shown to involve an initial hydrolysis to give a phenol, methylamine and carbon dioxide.¹¹⁶ These products could be used as a carbon and energy and/or nitrogen source, depending on the particular organism. The first *N*-methylcarbamate pesticide-hydrolyzing enzyme was described in 1987.¹¹⁷ In the same year, a phenylcarbamate herbicide-degrading enzyme was purified from a strain of *Pseudomonas alcaligenes*.¹¹⁸ This type of hydrolytic activity was demonstrated in 1988 in crude extracts of isolated carbofuran-degrading microorganisms.¹¹⁹ There was also evidence presented that in a few bacteria, carbofuran could be metabolized through an initial oxidative step. In 1989, the carbofuran hydrolase gene was cloned from an *Achromobacter* strain and characterized. Only very low levels of expression could be achieved at that time.¹²⁰ Mulbry and Eaton purified and characterized a hydrolase from a strain of *Pseudomonas*.¹²¹ In addition to carbaryl, it hydrolyzed carbofuran and aldicarb.

Table 8. Purified and Characterized Carbamate Enzymes.

Year	Organism	Substrates	MW (kDa)	Opt Temp (°C)	Ref.
1987	<i>Achromobacter</i> Strain WM111	Carbofuran Carbaryl Aldicarb	Dimer 150/70	50	122
1991	<i>Pseudomonas</i> Strain CRL-OK	Carbaryl Carbofuran Aldicarb	Dimer 187/85	60	123
1992	<i>Arthrobacter oxydans</i> P52	Phenmedipham Desmedipham	Monomer 55		124
1993	<i>Pseudomonas aeruginosa</i>	Carbaryl	Monomer 65	45	125
1993	<i>Blastobacter</i> Strain M501	Carbaryl 6 other pesticides	Dimer 166/84	45	126
2001	<i>Arthrobacter</i> Strain RC100	XMC Carbaryl Xylylcarb Metolcarb	Dimer 160/80	45	127
2002	<i>Pseudomonas</i> Strain 50432	Carbofuran Carbaryl	Monomer 88	37	128

In Table 8, the properties of these and other purified carbamate enzymes are summarized. As far as can be determined, none of these enzymes are currently available in large-scale quantities or commercially.

2.3 Sulfur Mustard and Organochlorine Pesticides.

Sulfur mustard, $(\text{ClCH}_2\text{CH}_2)_2\text{S}$, 2,2'-dichlorodiethyl sulfide, is one of the oldest of the classical chemical warfare agents and was used extensively in World War I, by the Italians in Ethiopia prior to World War II, and as recently as the Iran-Iraq War. It was actually first described as early as 1886.¹²⁹ Although frequently referred to as mustard gas, it is actually a heavy, oily liquid with the consistency of motor oil or Karo syrup. In addition to the names above it has also been known as Yperite, mustard; Schwefel-Lost; S-Lost; Lost; Yellow Cross liquid; Kampfstoffe; bis(2-chloroethyl) sulfide; and the acronyms SM, H and HD (for distilled mustard). In this report, it will be referred to as mustard. It should be noted that a number of nitrogen mustards were produced and weaponized by various nations. However, because these nitrogen mustards rapidly lose their toxicity in water due to hydrolysis, they most likely cannot be considered to be a significant threat for water supply systems.

Mustard is generally considered more of an incapacitating agent rather than an acutely lethal one. It is most known for causing blindness, large, long-lasting

blisters, and respiratory problems. Most fatalities from mustard were a result of severe pulmonary edema or infections of the blistered skin. It is able to alkylate a wide variety of naturally occurring functional groups of biologically important molecules such as, nucleosides, nucleotides, and other organophosphorus compounds (adenylic acid, glycerophosphate), RNA, DNA, amino acids, peptides and proteins. In particular, its ability to alkylate DNA is now believed to be the principal cause for its vesicant or blistering effects. An excellent review of mustard's properties was published in 1991.¹³⁰

The primary difficulty in dealing with mustard either enzymatically or chemically is its extremely low solubility in water. In very dilute solutions or at elevated temperatures, mustard will hydrolyze almost completely to the water miscible and non-toxic thiodiglycol (bis(hydroxyethyl)sulfide; TDG) and hydrochloric acid. The most commonly accepted path for this hydrolysis is shown in Figure 5. The difference in toxicity between mustard and TDG is a factor of 4,200 to 5,700 (the oral LD₅₀ of mustard is 0.7 mg/kg whereas that of TDG is 3000-4000 mg/kg¹⁰).^{131,132} The reduction in toxicity offers the potential for significantly reducing the damage caused by mustard if decontamination can be completed quickly enough.

With its extremely low water solubility and rapid hydrolysis at low concentrations, sulfur mustard, like the nitrogen mustards, would appear to be an unlikely candidate for contamination of water supply systems. Most decontamination systems (including enzymes) for mustard are aimed at dealing with contaminated surfaces or terrain.

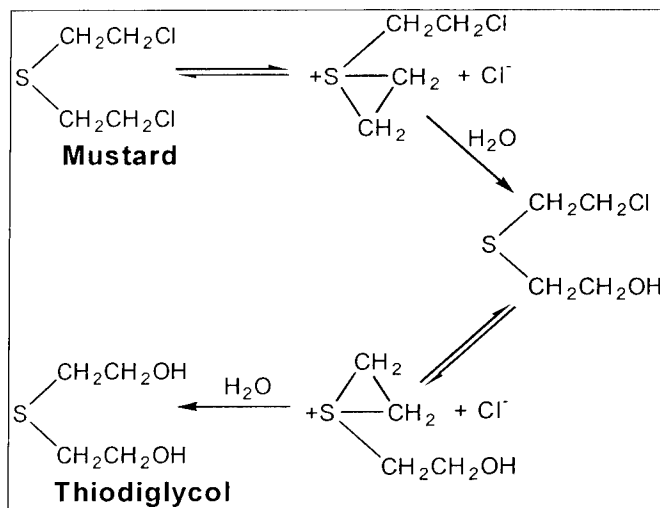


Figure 5. Hydrolysis Reactions of Mustard in Water.

Like V-agents, mustard is susceptible to oxidation at the sulfur to initially give mustard sulfoxide (HDSO), which is water soluble and quite stable. Further oxidation leads to mustard sulfone (HDSO₂) and potentially to cleavage of the C-S bond. However, those final steps require extremely powerful oxidation conditions. The two more common products are the sulfoxide and the sulfone. The sulfoxide is no longer a vesicant but does possess systemic toxicity and can cause injury to aquatic

life. However, the sulfone regains vesicant properties and has approximately the same toxicity as mustard itself.¹³³ For that reason, most oxidant-based decontaminants attempt to halt the reaction at the sulfoxide stage.

In the search for enzymes that could attack mustard, those that target organochlorine pesticides appeared to be good candidates. In addition, they could also help deal with these toxic and persistent pesticides. The major organochlorine pesticides generally fall into four classes: dichlorodiphenylethanes (e.g. DDT), cyclodienes (e.g. Aldrin, Chlordane, Dieldrin, Endrin, Heptachlor), chlorinated benzenes (e.g. Hexachlorobenzene [HCB], Pentachlorophenol [PCP]), and cyclohexanes (e.g. Hexachlorocyclohexane [HCH, Lindane]). These, plus Mirex, Toxaphene, PCB's, Dioxins and Furans, make up the United Nations Environment Program (UNEP) Persistent Organic Pollutant (POP) "Dirty Dozen." The danger of these materials was brought to public awareness in the 1962 by Rachel Carson's book *Silent Spring*.¹³⁴ Although many of these have been banned in the U.S. or greatly reduced in their use, some are still being produced in other nations. In addition, because of their persistency, they can be found in soil and sediments or concentrated in fatty foods, dairy products and fish.^{135,136} As an example, even though DDT was banned in the U.S. in 1973, it can still be found in the lipids of many adults.

Assuming that the solubility issues could be addressed, there are four possible mechanisms by which enzymes could potentially attack mustard. Three of these are shown in Figure 6. They are direct hydrolysis/dehalogenation, direct oxidation of the sulfur, or coupling chemical oxidation with enzyme hydrolysis/dehalogenation of the product(s). The fourth method would be to attack the C-S bond hydrolytically.

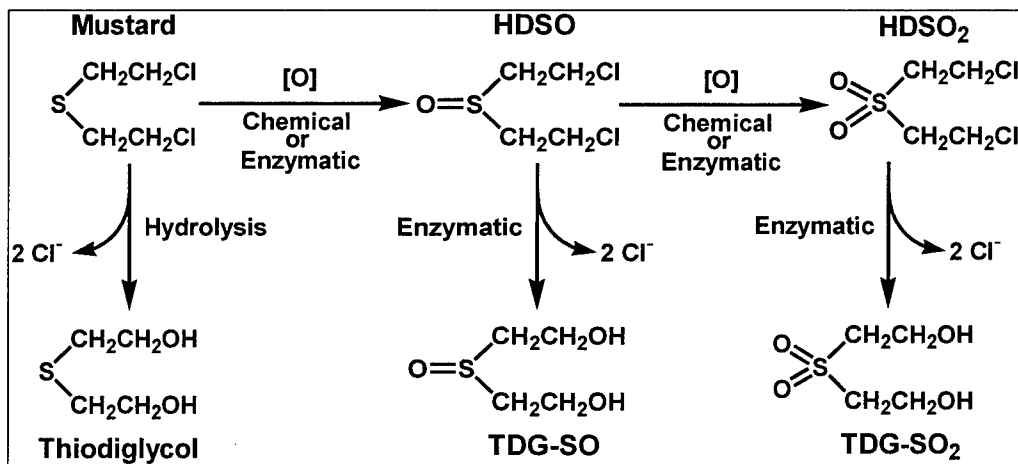


Figure 6. Enzymatic Methods for Decontamination of Mustard.

For many years, it was thought that direct hydrolytic dehalogenation by enzymes was unlikely, if not impossible. However, in the past few years, several groups have shown that bacterial haloalkane dehalogenases can catalytically react with mustard.^{137,138} However, this can only be observed with vigorous stirring and when the

mustard is supplied as a solution in alcohol. In this manner, the mustard is efficiently dispersed and significant enzyme enhancement of hydrolysis (as measured by chloride release) can be observed. If the same quantity of mustard is added as a neat liquid, little or no activity is seen. As yet, nothing has been identified that will eliminate the problem without the use of high levels of organic solvents. Low organic content microemulsions may provide a means of overcoming this problem, but they will also have to be compatible with the enzymes. Table 9 gives some partial list of some of the bacterial hydrolytic dehalogenases (halohydrolases) that have been described in the open literature. As can be seen, they are capable of dealing with a wide variety of substrates.

Since these enzymes are single polypeptide chains and do not require cofactors they are attractive candidates for use in decontaminating mustard and the chlorinated pesticides. However, as with the carbamate degrading enzymes, none of these are currently commercially available.

An alternative hydrolytic enzyme to the dehalogenases is known. A recent report describes how in *Sphingobium chlorophenolicum* the initial removal of chlorine in pentachlorophenol (PCP) is through NADPH-dependent hydroxylation by PCP hydroxylase.¹³⁹ Although the microorganism is potentially of interest for PCP biodegradation either *in situ* or in bioreactors, a cofactor-requiring enzyme has limited utility in cell-free decontamination systems.

In a paper describing the degradation of mustard by lignin-degrading fungi, another possible hydrolytic mechanism was observed.¹⁴⁸ Unlike the other hydrolytic enzymes discussed, there appears to be direct hydrolysis of the C-S bond of mustard to yield chloroethanol and chloromercaptoethane. These products were then dechlorinated to give mercaptoethanol and ethylene glycol, both of which were then completely metabolized. Unfortunately, the enzyme responsible for the C-S bond cleavage has not been characterized, so it is unknown whether it could play a role in the development of decontamination systems.

An oxidative enzyme capable of acting on halogenated phenols is a dehalogenating peroxidase isolated from a marine polychaete. As reported by Chen et al.,¹⁴⁹ the enzyme for *Amphitrite ornate* is active against mono-, di- and trihalogenated phenols with bromo-, chloro-, and fluoro-substituents. This small (MW 30.8 kDa, with two identical subunits, MW 15.4 kDa), heme-containing enzyme appears to be related to globins based on its amino acid sequence.¹⁵⁰ Since peroxide can be enzymatically generated *in situ* (ex. Glucose Oxidase or Alcohol Oxidase), this type of enzyme could potentially be useful in a decontaminant. However, it is not yet known whether it will attack phenol pesticides such as PCP, the other varieties of chlorinated pesticides or mustard.

Table 9. Purified and Characterized Hydrolytic Dehalogenases.

Year	Organism	Primary Substrates	MW (kDa)	Ref.
1985	<i>Xanthobacter autotrophicus</i> Strain GJ10	1,2-Dichloroethane 1,2-Dibromoethane	Monomer 36	140
1987	<i>Rhodococcus</i> Strain m15-3	1,2,3-Trichloropropane 1,2-Dibromo-3-chloropropane 1,2-Dibromoethane	Monomer 33	141
1987	<i>Rhodococcus</i> Strain G70	1,2 Dibromoethane Ethylbromide 1-Bromopropane 1,3 Dichloropropane 1,6-Dichlorohexane 1,9-Dichlorononane	Monomer 33	142
1989	<i>Sphingomonas paucimobilis</i> Strain UT26	α , δ , γ -HCH (Lindane)	Monomer 30-32	143
1990	<i>Sphingomonas paucimobilis</i> Strain B90	α , β , δ , and γ -HCH (Lindane)	Monomer 30-32	144
1993	<i>Ancylobacter aquaticus</i> Strains AD25 and AD27	2-Chloroethylvinylether 2-Chloroethylmethylether 2-Bromoethylethylether		145
1994	<i>Rhodococcus rhodochrous</i> NCIMB 13064	1,2-Dibromoethane 1-Chlorobutane 2-Chlorobutane	Monomer 33	146
1996	<i>Mycobacterium</i> Strain GP1	2-Bromoethanol 1,2-Dibromoethane Bromochloroethane	Monomer 33	147

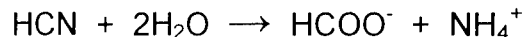
There are numerous chemical oxidants capable of oxidizing mustard to its sulfoxide or sulfone. However, very little work has been reported on enzymatic oxidation until recently. In 1990, Colonna et al. described the ability of a chloroperoxidase from the marine fungus *Caldariomyces fumago* to catalyze the oxidation of a wide variety of sulfides (although not mustard).¹⁵¹ More recently, Amitai has demonstrated the ability of fungal laccases and haloperoxidases to rapidly oxidize both VX¹⁵² and mustard.¹⁵³ The primary problem with these enzymes is that they normally function best at acidic pH values (3 to 5). However, Amitai¹⁵⁰ has shown that the haloperoxidases can react with sodium chlorite at neutral pH to generate chlorine dioxide, which is a powerful oxidant. Not only was the enzymatically generated chlorine dioxide as much as 10 times more effective than added it directly, it was also significantly less corrosive.

As noted above, chemically or enzymatically oxidizing mustard to its sulfoxide or sulfone does not solve the toxicity problems. Elashvili¹⁵⁴ has determined that both the mustard sulfoxide and sulfone can be dechlorinated by the hydrolytic

dehalogenase from *Sphingomonas paucimobilis* UT26 to their respective thiodiglycol derivatives that are relatively non-toxic. It is presumed that other hydrolytic dehalogenases will be able to carry out this reaction (especially those from the *Rhodococcus* strains), however this has not yet been reported.

2.4 Cyanide.

In addition to their potential use as chemical warfare or terrorist agents, cyanide compounds are extensively used in industry and are also found in effluents from food and feed production. Microorganisms are known to possess a variety of different enzyme systems capable of degrading cyanide, but because of the intent of using cell-free enzymes in decontamination, those that require cofactors (such as cyanide oxygenase¹⁵⁵) will not be considered in this review. In 1991, a very promising enzyme from a strain of *Alcaligenes xylosoxidans* that converted cyanide to ammonia and formate in a one-step reaction, was described by researchers at Novo Nordisk.¹⁵⁶



This reaction was much more attractive than those involving fungal decomposition of cyanide.¹⁵⁷ These systems involve a nitrilase-type enzyme that takes cyanide to an intermediate product formamide that is then converted to carbon dioxide and ammonia by a second enzyme, formamide hydrolyase.



The following year Novo Nordisk began commercial production of their enzyme under the name CYANIDASE[®]. To reduce cost, the enzyme preparation was as granulated whole cells with no attempt to purify the enzyme. In addition, since the enzyme was composed of at least eight identical (or four each very similar) subunits, using the whole cell system generally improves long-term stability. The potential use of the granulated CYANIDASE[®] in cleaning wastewater was reported in 1992. Basheer et al. showed that the preparation could reduce CN⁻ to very low levels in wastewater (0.01-0.02 ppm).¹⁵⁸ Unfortunately, Novo Nordisk (now Novozymes) discontinued the production of CYANIDASE[®] in the late 1990's and it is no longer available.

An enzyme that carried out the same reaction as CYANIDASE[®] was isolated from *Bacillus pumilus* C1 and designated as a cyanide dihydratase.¹⁵⁹ Although it also converted cyanide directly to ammonia and formate, it was clearly a different enzyme. It has a significantly higher temperature optimum (37 °C versus 26 °C) and is composed of at least three each of three different subunits, the NH₂-terminal sequence of which bear no similarity to the *Alcaligenes* enzyme.

Also in the early 1990's, a cyanide dihydratase (or nitrilase/hydratase) was tentatively identified in a *Pseudomonas fluorescens* strain, but as yet, it has not been purified or characterized.^{160,161}

More recently, another cyanide dihydratase (or cyanidase) was purified and characterized from *Pseudomonas stutzeri*.¹⁶² Unlike the earlier enzymes, the gene for this cyanidase was cloned and expressed at high levels in *E. coli*.¹⁶³ While it has some similarities to the enzymes from *B. pumilus* and *A. xylosoxidans* (similar size and K_m for cyanide), it is also different in a number of properties. The NH₂-terminal sequence shows considerable homology to that of *A. xylosoxidans* but it is composed of a single polypeptide, whereas the others have two or three different ones. *B. pumilus* cyanidase is significantly stimulated by the addition of Cr³⁺, whereas *P. stutzeri* is unaffected. In addition, the temperature optimum of *P. stutzeri* is intermediate to the other two enzymes at 30 °C. As with the original CYANIDASE®, this enzyme would appear to have considerable potential. However, its potential availability remains unknown. In summary, the ability to effectively deal with cyanide contamination appears to be quite promising if this and related enzymes would be available in sufficient scale.

2.5 Biological Agents.

For the purpose of this report, biological agents, whether for biological warfare (BW) or natural pathogens, will be considered "living" organisms. They will be collectively referred to as biological agents. Toxins will be dealt with in a separate section. The types of biological agents that could pose a threat to water supply systems include bacteria, rickettsiae, protozoa and viruses. Some would be potentially lethal; others would be more incapacitating in nature. The following Table¹⁶⁴ is the list of Select Agents whose shipment is regulated by the CDC under the Antiterrorism and Effective Death Penalty Act of 1996. It should be noted that for a number of these, the vaccine strains are exempt. The bottom section of the table has additional, non-regulated biologicals that would also be threats to water supplies.

The destruction of biological agents can be achieved by a variety of methods. This review will not deal with chemical disinfectants or decontaminants unless they are produced biologically. Antibiotics and antimicrobial peptides (AMPs) are two of the major biotechnology-based means of dealing with bacterial pathogens. Antibiotics alone could be the basis for a huge literature review, but their use would be unlikely for dealing with contaminated water supplies or facilities. Over the past 25 years, a great deal of attention has been given to AMPs that are produced by a wide variety of organisms, up to and including humans. The principal effect of AMPs is to bind to and produce holes in cell membranes leading to cell lysis. Intended primarily for *in vivo* treatment or food preservation, AMPs show a greatly reduced incidence of resistance being developed by bacteria in comparison to antibiotics. The variety of AMPs is quite diverse with more than 800 being described so far. While quite effective, AMPs are not catalytic in nature and will not be discussed further in this review. Additional information on AMPs and their properties can be obtained in a variety of recent publications.¹⁶⁵⁻¹⁶⁸

Table 10. Biological Agents.

<u>Viruses</u>	<u>Bacteria</u>
Crimean-Congo hemorrhagic fever virus Eastern equine encephalitis virus Ebola virus Equine morbillivirus Lassa fever virus Marburg virus Rift Valley fever virus S. American hemorrhagic fever viruses (Junin, Machopa, Sabia, Flexal, Guanarito) Tick-borne encephalitis complex viruses Variola major virus (smallpox virus) Venezuelan equine encephalitis virus Viruses causing hantavirus pulmonary syndrome Yellow fever virus	<i>Bacillus anthracis</i> <i>Brucella abortus</i> , <i>Brucella melitensis</i> , <i>B. suis</i> <i>Burkholderia (Pseudomonas) mallei</i> <i>Burkholderia pseudomallei</i> <i>Clostridium botulinum</i> <i>Francisella tularensis</i> <i>Yersinia pestis</i>
	<u>Rickettsiae</u> <i>Coxiella burnetii</i> <i>Rickettsia prowazekii</i> <i>Rickettsia rickettsii</i>
	<u>Fungi</u> <i>Coccidioides immitis</i>
<i>Clostridium perfringens</i> <i>Cryptosporidium parvum</i> <i>Giardia lamblia</i>	<i>Salmonella</i> spp. <i>Shigella</i> spp. <i>Vibrio cholerae</i>

One of the earliest enzymes known to have antibacterial action is lysozyme. It was discovered in 1921 in nasal mucus by Alexander Fleming who five years later discovered penicillin.¹⁶⁹ Unfortunately, it did not turn out to be the medical antibiotic he was searching for, nor did it have very good activity against pathogens. However, lysozyme (now primarily extracted from hen egg whites) has been used ever since in disrupting *E. coli* and other bacteria in research labs. A small globular protein containing 129 amino acids was also the first enzyme ever to have its structure solved. Lysozyme's active site cleaves the bond between the N-acetylmuramic acid (NAG) and N-acetylglucosamine (NAM) monomer units of the peptidoglycan found in the cell walls of some Gram-positive bacteria. This mainly occurs during the growth of the cells, with much less activity when the bacteria are dormant. The relative hydrolytic rate of lysozyme is slow, but because of the high osmotic pressure within bacteria cells, only a few "nicks" in the cell wall cause the bacteria cell to rupture. In a 1986 report, Singh and Doyle reported on the effect of a mixture of lysozyme and mutanolysin (an extracellular enzyme from *Streptomyces globisporus*¹⁷⁰) on a variety of *Bacillus* species.¹⁷¹ Mutanolysin had previously been shown by them to be active against *Bacillus anthracis* and vaccine strains of *Brucella abortus*, *Brucella melitensis*, *Francisella tularensis* and *Yersinia pestis*. While several of the *Bacillus subtilis* strains were rapidly destroyed, the other species were affected to some degree, but without complete lysis. A combination of pepsin and lysozyme did not yield significant lysis. Both lysozyme and mutanolysin are commercially available.

In a later study conducted at ECBC, both lysozyme and mutanolysin were tested separately against non-pathogenic strains of *Bacillus anthracis*.¹⁷² Lysozyme inhibited the growth of one of six *B. anthracis* strains, while mutanolysin was active against three strains. The surprise of the study was with the enzyme chitinase (from *Streptomyces griseus*), which was active against all six strains. Chitin, a homopolymer of N-acetyl-D-glucosamine (Glc-Nac) residues linked by β 1-4 bonds, is the most abundant renewable natural resource after cellulose.¹⁷³ It is widely distributed in nature with most of it found in the exoskeletons and endoskeletons of many organisms including mollusks, crustaceans, insects, protozoa, and fungi. Many plants as well as some animals produce chitinases as a means to provide protection against fungal infections. Numerous microorganisms also produce an array of chitinases. Since the cell walls of bacteria do not have chitin, chitinase was not expected to have antimicrobial activity. However, we then learned that some animal and higher plant chitinases also have lysozyme activity.¹⁷⁴ In 1997, bifunctional chitinases/lysozymes were identified in a strain of *Pseudomonas aeruginosa*.¹⁷⁵ They showed broad lytic activity against both Gram-positive and Gram-negative bacteria. While there has been considerable interest in the use of chitinases as insecticides, very little attention has been paid to their bactericidal properties. A number of chitinases (primarily bacterial) are commercially available.

To further enhance their bactericidal activities, lysozyme, mutanolysin and chitinase can be combined into a cocktail with other enzymes (proteases and lipases) as well as detergents/surfactants that will disrupt cell membranes.

The enzymes and approaches described above are relatively generic in nature (especially the enzyme cocktail). However, in some circumstances when the specific pathogen is known, it may not be necessary to sterilize the material being treated. Recently, enzymes have been described that permit the targeting of specific bacterial species. These are enzymes produced by lytic bacteriophage. Bacteriophage or phage, are viruses that infect bacteria and were first discovered nearly 90 years ago.^{176,177} They can be extremely specific in regards to what bacteria they will infect, down to the species or even strain level. It is now believed that at least some phage have two types of lytic enzymes, to either allow them to introduce their genomes into host cells (lytic transglycosylases),¹⁷⁸ or to break open the host cell to release phage progeny (lysins).¹⁷⁹ While there has been a great deal of work beginning in the 1920's dealing with the use of bacteriophage for antibacterial therapy, it was only recently that the use of phage enzymes was actively considered as potential treatments or decontaminants. Fischetti and his group at The Rockefeller University have been leading these efforts. In 2002, he reported on ability of the lysin from a dsDNA phage to selectively and rapidly kill vegetative cells of *Bacillus anthracis*.¹⁸⁰ They used the γ phage since it is known to infect nearly all *B. anthracis* strains and a few rare and closely related *B. cereus* strains. The recombinant γ lysin (called PlyG, for phage lysin γ) is a single polypeptide of 27-kDa molecular weight. In addition to *B. anthracis*, they have identified lysins against *Streptococcus pneumoniae* and staphylococcal pathogens.¹⁸¹ Another group has cloned and characterized a similar lysin from a phage that is specific for (which causes gas gangrene or necrotic enteritis).¹⁸² Like the phage

itself, the lysin (Ply3626) only attacks strains of *C. perfringens*. Although there is considerable interest in the lysins by pharmaceutical companies and the military, it is not believed that they are commercially available yet.

In dealing with biological agents, the most challenging targets are spores. Although most of the attention has recently been given to *B. anthracis*, cysts of the freshwater pathogens *Cryptosporidium parvum* and *Giardia lamblia* are equally, if not even more difficult to kill. In the case of *B. anthracis*, one approach that is being taken is to incorporate germinants (ex. glucose, L-alanine, adenosine, inosine, etc.) into decontamination formulations. By forcing the spores to germinate, they lose their protective properties and are susceptible to attack by both chemical and biological means. However, it would be greatly preferable if one could attack the spores directly. One obvious approach that is being examined is to use the same enzymes that the spore uses during germination to attack it from the outside. One such enzyme from *B. cereus* has been identified and shown to be able to attack the spore core, but only if the spores were stripped of the coat fraction.^{183,184} Other lytic enzymes from *Bacillus subtilis* have been identified, but as yet there are no reports on their ability to react with either intact or coat-stripped spores.^{185,186}

The last type of enzyme for biological agents to be discussed involves the non-specific generation of disinfectant materials. The most common enzymes in this category are peroxidases or haloperoxidases that use hydrogen peroxide to activate halides to produce antimicrobial compounds. While hydrogen peroxide itself is a relatively good disinfectant, it often is not a strong enough oxidant to deal with microbial spores or protozoan cysts. The actual mechanism can take several forms depending on the type of halogen/halide used. In some systems, it is thought to be hypohalous acid (HOX) that is formed and carries out the attack on the pathogen.¹⁸⁷ Alternatively, the HOX may react with peroxide to generate singlet molecular oxygen on the surface of the target microbe.¹⁸⁸ It has been known since 1967 that the enzymatic reaction of chloride ions and hydrogen peroxide gave antimicrobial results.^{189,190} The enzymes that have been shown to carry out this reaction include myeloperoxidases,¹⁹¹ eosinophil peroxidase,¹⁹² lactoperoxidase, horseradish peroxidase, haloperoxidases and catalase.¹⁹³⁻¹⁹⁶ This enhanced hydrogen peroxide killing has been demonstrated with bacteria, yeasts, fungi, human immunodeficiency virus type 1, the parasitic helminth *Schistosoma mansoni* (see references in ¹⁹⁷) and cysts of the amoebae *Acanthamoeba*.¹⁹⁸ As mentioned above in the section dealing with oxidation of mustard, some of the peroxidases can react directly with sodium chlorite (NaClO_2) to form the very potent disinfectant chlorine dioxide (ClO_2) which is effective in both liquid and gas form.

2.6

Toxins.

Protein toxins such as botulinum and ricin are among the most toxic substances known (see Table 1). However, there is a vast array of commercially available proteases that, singly or in combination, could convert them to harmless peptides or even free amino acids. If it is known that only protein toxins are the targets, such a formulation could be easily developed. However, if the proteases are to be combined with other degradative enzymes the challenge becomes much greater. In that type of situation, it would be necessary (or at least desirable) to employ proteases that could selectively cleave the toxins, but leave the other enzymes unaffected. Because of the incredible diversity in protease specificity, this is certainly achievable, especially with new methods of high throughput screening to simplify the process. However, this reviewer is not aware that any such study has yet been undertaken. In such a study, if one or more proteases had the desired specificity, but another enzyme in the mixture is attacked, this could be dealt with through advanced techniques in molecular biology. Mutants of the affected enzymes could be developed that would still be active, but with the susceptible amino acid sequences modified to eliminate attack by the specific protease(s). Identifying this modification/mutation should not be a limiting factor since any enzymes use in a decontamination formulation will already be cloned, sequenced and expressed in order to produce sufficient quantities.

The situation with non-protein toxins (mycotoxins, tetrodotoxin, saxitoxin, palytoxin, etc.) is almost entirely the reverse. Potentially, because of their toxicity and chemical stability, a very large number of these toxins could be threats. To further complicate the situation, they vary tremendously in structure from one another. Therefore, it is extremely unlikely that specific decontaminating enzymes will be used to deal with them. Much more likely would be to use relatively non-specific enzymes, such as some that have already been described in this review. These would include laccases, peroxidases, and haloperoxidases that might be coupled with peroxide generating enzymes (oxidases). The decontamination or inactivation of the toxins would come from the oxidizing effects of the hydrogen peroxide, hypohalous acids, chlorine dioxide, or other reactive species that would be generated. With some of the toxins, modifying a single group will dramatically change its biological activity. These enzyme-generated oxidants may also be active against the protein toxins, but that remains to be verified.

2.7

Biofilms.

For more than a century, microbiologists have chosen to study bacteria that grow in suspended culture, primarily for simplification of experimentation and the homogeneity of conditions. However, it has become more and more obvious that in natural environments, microorganisms grow in aggregation with each other on solid surfaces or at gas-liquid interfaces. While use of biofilms is desirable in some applications such as wastewater treatment or removal of volatile organic compounds (VOC's) from air streams (ex. trickling filters), they are generally to be avoided. Much more attention is being paid to these aggregations or biofilms as the problems they can

cause is recognized. These can include increased frictional resistance to fluids in water conduits and on ship hulls (fouling),¹⁹⁹ decreased heat transfer from heat exchangers,²⁰⁰ corrosion of metallic substrata,²⁰¹ and contamination of food and biotechnology industries. In addition, they are a severe problem in medical science and industry, causing dental plaque,²⁰² contaminated endoscopes and contact lenses,²⁰³ prosthetic device colonization, and biofilm formation on medical implants.²⁰⁴ For the purpose of this review, they can also provide a reservoir for chemical and biological threat materials and prevent their destruction by sequestering them from decontaminating enzymes.²⁰⁵ This can lead to a slow release of these highly toxic or dangerous materials, even after decontamination has been "completed."

The biofilm matrix is a collection of microcolonies with water channels in between and an assortment of cells and extracellular polymers (polysaccharides, glycoproteins, and proteins).²⁰⁶⁻²⁰⁹ Because of the variety of sugars and sugar-derivatives and the different types of bonds used, this gives rise to a multitude of polysaccharides, including levans, polymannans, dextrans, cellulose, amylopectin, glycogen, and alginate.

Individual enzymes have been used for the degradation of biofilms,²¹⁰⁻²¹³ but as would be expected a mixture of enzymes will work more efficiently. Enzyme mixtures were examined in a 1997 report.²¹⁴ Glucose oxidase combined with lactoperoxidase was bactericidal against biofilm bacteria, but did not remove the biofilm from the substrata. A complex mixture of polysaccharide-hydrolyzing enzymes (Pectinex Ultra SP) was able to remove bacterial biofilm from steel and polypropylene substrata but did not have a significant bactericidal activity. Combining oxidoreductases with polysaccharide-hydrolyzing enzymes resulted in bactericidal activity as well as removal of the biofilm.

In 2003, a group in the United Kingdom evaluated a variety of disinfectant products against a mixed-species biofilm.²¹⁵ The only enzyme-based disinfectant tested (of 23), Combizyme, a mixture of carbohydrases and proteases did not completely eliminate the viable bacteria nor remove the biofilm. Since this formulation did not include oxidoreductases, that could explain its poorer performance.

Somewhat in contradiction to this was another 2003 study²¹⁶ in which the use of lysozyme was effective in removal of a biofilm of the common thermophile *Geobacillus stearothermophilus* and preventing its reattachment. However, this may not be applicable for mixed-species biofilms.

A study in Finland in 2004 examined 16 different commercial enzyme-based products for their ability to clean and disinfect surfaces contaminated with *Pseudomonas aeruginosa*, *Lactobacillus bulgaricus*, *Lactobacillus lactis* and *Streptococcus thermophilus* biofilms.²¹⁷ Of the enzymatic cleaning products, the ones that were most potent in 30 minutes against the bacterial biofilms were Pandion, Resinase A2X, Spezyme GA300 and Paradigm. Only Resinase A2X and Paradigm showed a good effect on the biofilms after a 15-minute incubation time. Of the

disinfectants, proteinase ones (alkalase, chymotrypsin, cryotin and krilltrypsin) showed a good effect on the *P. aeruginosa* biofilm, but the effects were reduced in the presence of competing materials such as milk.

As a gauge of the interest in biofilms, a recent conference sponsored by the American Society for Microbiology, Biofilms 2003, had 638 participants from 36 countries.²¹⁸ One of the keynote speakers, J. William Costerton, is Director of the Center for Biofilm Engineering in Bozeman, MT.

As has been noted with other potential threats, the enzymatic destruction of biofilms will most efficiently be achieved through mixtures that act synergistically to far exceed the capabilities of any one single enzyme.

3. STABILIZATION AND IMMOBILIZATION METHODS

The unique catalytic properties of enzymes led to their rapid exploitation in the food industry,²¹⁹ analytical chemistry,²²⁰ preparative organic chemistry,²²¹ medicine,²²²⁻²²³ etc. Moreover, of course, the whole field of genetic engineering (recombinant DNA technology) would not have been possible without the use of enzymes. However, it quickly became apparent that there were limitations to this technology owing to the denaturation or inactivation of enzymes brought about by heat, proteolysis, action of organic solvents, etc. There was a major incentive to find solutions to these problems in order to take advantage of enzymes active, specificity and other attractive features. Even in the early 1970's there were already several good reviews on the development of the new biochemistry based on immobilization procedures.²²⁴⁻²²⁶

Trying to review the entire field of enzyme immobilization over the past 40 years could easily triple or quadruple the size of this document. Therefore, it will concentrate on some of the methods that may prove most useful in dealing with contaminated water and water supply systems. The initial system to be addressed in this review is the development of an enzyme filter through which contaminated water can pass. This application is much closer to those used in the majority of immobilized enzyme systems-whether for degrading or synthesizing chemical species.

3.1 Immobilization Methods.

The various methods for enzyme immobilization can be classified into three basically different approaches as illustrated in Figure 7. They are the support-binding method, the cross-linking method and the entrapment. As the technology advanced, combinations of these methods have also been employed. For all the methods that will be discussed in this review, it should be recognized that none are 100% effective with all enzymes. Only by testing the methods with the particular enzymes in question can one be certain of their efficacy.

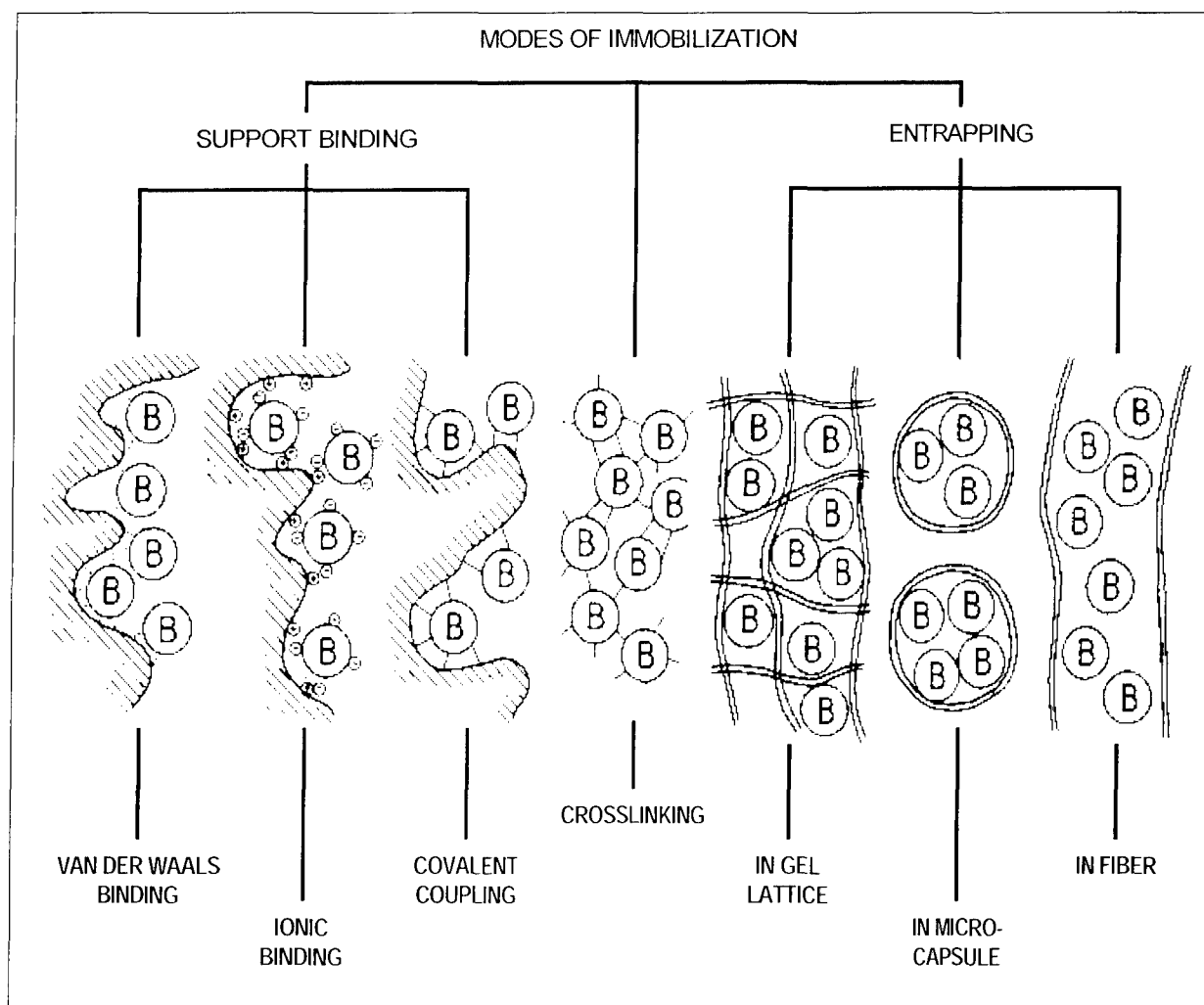


Figure 7. Classical Methods for the Immobilization of Enzymes.²²⁷

The binding of enzymes to solid supports is the oldest immobilization technique. The number of published papers on this type of immobilization alone probably numbers in the thousands. Perhaps the simplest of all the techniques and one that does not grossly alter the activity of the bound enzyme is adsorption through ionic interactions on an ion exchanger.²²⁸ The potential reversibility of binding by changing the pH or ionic strength of the surrounding medium allows for economic recovery of the support when the enzyme activity has dropped to below operational parameters. In some instances, the enzymes will first be bound to the support through ionic binding and then fixed in place by intermolecular cross-linking.

Another adsorption method not illustrated in Figure 7 is hydrophobic interaction. This technique is most useful at high ionic concentrations, which are desirable and important in enabling the use of high concentrations of substrates commonly encountered in industrial operations. As with ionic binding the substrate can be recovered after the enzyme activity has been spent by lowering the ionic strength of

the medium - assuming that the enzymes have not been covalently cross-linked after binding. With the much lower ionic strength of water supply systems, this technique would not be appropriate.

By far the most common means of binding to solid supports involves covalent coupling. Many different functional groups on the enzymes are used for the covalent attachment, including amino, carboxy, sulphhydryl, and the phenolic group of tyrosine. No matter which functional group(s) on the enzymes are used for covalent linkage, it is essential that they not be involved in the catalytic activity. In order to avoid this, conducting the immobilization in the presence of a substrate or competitive inhibitor is often employed. By doing so, the active site of the enzyme may be protected. However, changing the conformational flexibility of the enzyme also needs to be avoided since that can significantly alter its activity.

If the enzyme is from a eukaryotic source (fungi, yeast, animals, etc.), it very possibly may have carbohydrate attached as a result of post-translational modification. These glycoproteins can often be immobilized through binding to the carbohydrate without causing an alteration of catalytic activity. This is done through introducing an aldehyde group in the carbohydrate moiety by periodate oxidation treatment. The modified enzyme can then be covalently linked to a support containing an alkyl amine group through Schiff's base reaction. The Schiff base intermediate is then reduced (for example with sodium borohydride, NaCNBH_3) to give a stable secondary or tertiary amine linkage.²²⁹

For some enzymes, intermolecular cross-linking the enzyme molecules by means of multifunctional reagents in the absence of a solid support can produce a water-insoluble preparation. Glutaraldehyde is one of the more commonly used cross-linking agents since it has achieved GRAS (generally regarded as safe) status, is low-cost, has high efficiency, and the cross-links are quite stable. In some cases, to improve stability, a low cost inert protein like gelatin, albumin or collagen may be included. Both cross-linking and support binding may be most effective when dealing with high molecular weight substrates. While cross-linking can yield a biocatalyst with very high specific activity (since it is only composed of enzyme molecules), it is often not sufficiently robust for large-scale or long-term use.

Entrapment is the method that has had the greatest use in large-scale enzyme operations. Since most enzymes used commercially are intracellular, the simplest method, which is not shown in the figure, is to use whole cell preparations where the enzyme is never released from the bacteria in which it is produced. One of the best examples of this is glucose isomerase, which has been used in the commercial production of high fructose corn syrup (HFCS) since 1967.²³⁰ Most of the glucose isomerase used in the production of over 6,000,000 tons of HFCS per year is in the form of immobilized whole cells.²³¹ This is often done by spray drying the harvested cells to give a granulated product that is then treated with polymeric materials (such as polyethylenimine) to stabilize them. To further improve reactivity, the cells may be permeabilized. This removes the barrier for the free diffusion of the substrate/product

across the cell membrane, and also empties the cell of most of the small molecular weight cofactors, etc., thus minimizing unwanted side reactions. Obviously, this and all other entrapment techniques are most applicable for low molecular weight substrates and simple bioconversions like hydrolysis, isomerization and oxidation reactions that do not require a cofactor-regeneration system.²³²

It is possible to use entrapment as a mean of immobilizing a cofactor requiring system. This can be accomplished in several ways. Initial efforts involved covalently attaching the cofactor (usually NAD or NADH) directly onto a solid support or in a gel matrix along with the enzymes.²³³ However, while the enzymes and cofactor would be stable, the level of activity was generally quite low. Other researchers attached the cofactor directly to the enzyme by means of a bifunctional linker such as modified polyethylene glycol (PEG). The linker would ideally be short enough to keep the cofactor close to the active site. However, it would also need to be long and flexible enough to permit its easy entrance in and out of the active site.²³⁴ The most commonly used method has been to attach the cofactor to PEG or other large polymer. This is then placed with the enzyme(s) in ultrafiltration systems with a semipermeable membrane, microencapsulated, or immobilized in membranes. In most cases, the cofactor would be regenerated with a second enzyme such as formate or alcohol dehydrogenase, for which a second substrate is required. When the process is being used for the synthesis of a valuable product, this can be cost effective. However, for use in biodegradation such an approach would be too expensive. Two enzymes described recently could simplify the system and reduce costs. The first involves a hydrogenase that in the presence of hydrogen can regenerate NADH.²³⁵ This is still somewhat involved since hydrogen would need to be provided. An even more interesting enzyme is an NADH Oxidase that can use dissolved oxygen to regenerate NAD with the production of water.²³⁶ Thus, only aeration of the system would be required and no unwanted products would be generated.

While entrapment in a gel matrix has been extensively used for the immobilization of cells, it has not been as common for free enzymes. The major limitation of this technique for enzymes is the possible slow leakage during continuous use.²³⁷ The primary natural polymers used for entrapment have been agar, agarose and gelatin through thermoreversible polymerization, and alginate and carrageenan by ionotropic gelation. In addition to possible enzyme leakage, these are relatively soft materials that will deform in large packed columns. They are also subject to deterioration if used in fluidized bed reactors. In the case of alginate and carrageenan, the ionic species used in the polymerization (usually Ca^{2+}) needs to be present continuously to maintain the integrity of the gels. With some enzymes and processes, this can be a problem.

A wide variety of synthetic polymers have also been used to entrap both cells and enzymes. Early on, acrylic polymers such as polyacrylamide²³⁸ were very widely used, although not in the food industry where its toxicity was an issue. Since the production polymers such as polyacrylamide, polyurethane and photo-crosslinkable resins also can result in covalent attachments to the enzyme molecules; leakage from

these types of gels is virtually eliminated. The properties of the gels (porosity, density, hardness, etc.) are much easier to modify or tailor than with the natural polymers. In recent years, Alan Russell's group at the University of Pittsburgh has conducted the leading work in the use of polyurethane foams for enzyme entrapment. Of relevance to this project, he has done considerable work on immobilizing DFPase, OPH and OPAA enzymes. Although OPAA turned out to be one of the few enzymes incompatible with this technique, DFPase and OPH have been very successfully immobilized and shown to have significant increases in stability.²³⁹⁻²⁴² B.P. Doctor and his group at Walter Reed Army Institute of Research (WRAIR) have followed on this work with the immobilization of cholinesterases.²⁴³⁻²⁴⁴ As mentioned in an earlier section above, the use of a substrate or competitive inhibitor may enable the use of this technology for OPAA as well.

In addition to the type of synthetic polymers commonly thought of in regards to enzyme entrapment (polyacrylamide and polyurethane) some enzymes have been entrapped in plastics such as polymethylmethacrylate, polystyrene, polyvinyl acetate, and polyethylvinylether. To achieve this type of immobilization, the enzymes need to have significant resistance to the organic solvents used in the polymerization process. Both subtilisin Carlsberg and α -chymotrypsin have been incorporated into these types of plastics to give very stable biocatalysts.²⁴⁵

One of the problems with entrapment can be diffusional limitations as well as steric hindrance, especially when the substrates are large macromolecular materials such as starch and proteins. Diffusional limitations can be reduced by entrapping the enzymes in very fine fibers or by using an open pore matrix, such as is possible with polyurethane. Two types of entrapment that have been getting a great deal of attention recently are hydrogels and sol-gel. Hydrogels involve the use of thermoreactive water soluble polymers, like albumin-polyethylene glycol or *N*-isopropylacrylamide/acrylamide copolymer that can have water contents of up to 96% and provide a microenvironment for the immobilized enzyme close to that of the soluble enzyme with minimal diffusional restrictions.^{246,247}

While glass or silica beads have been used as solid supports for enzymes for many years, over the past eight to ten years a different type of immobilization has emerged – entrapment in silica or titania sol-gels. The typical sol-gel process developed in 1992 involves the preparation of an appropriate alkoxide that is mixed with water and an acidic catalyst such as HCl. After sonication to produce silanol groups (Si-OH) the sol is added to a buffer solution. The enzyme solution in buffer is then added to the buffered sol and the solvent (water) slowly removed.²⁴⁸ The final product is a rigid transparent material with good thermal and dimensional stability and with fine pore networks (<100 Å) that allow the diffusion of small molecules. For immobilization of enzyme on optical sensors there is the added benefit that the pore network is small enough that it does not scatter visible light. A considerable number of enzymes, including OPH,²⁴⁹ have been immobilized on alumina,²⁵⁰ silica²⁵¹⁻²⁵⁹ or titania^{260,261} sol-gels using this procedure. However, for some enzymes/proteins, this technique is still too harsh. Recently, a very novel, biotechnology-based method for

entrapping enzymes in silica has been developed.²⁶² This method uses the same system found in diatoms for creating their exoskeletons. It involves a solution consisting of silicic acid (hydrolyzed tetramethyl orthosilicate) and a silica-condensing synthetic peptide (derived from the silaffin polypeptide found in the diatom *Cylindrotheca fusiformis*). The peptide catalyzes the precipitation of silica within seconds when added to a solution of silicic acid.²⁶³ The resulting material is a network of fused spherical silica particles with an average diameter of 500 nm. Using the enzyme butyrylcholinesterase, 90% of the initial free enzyme activity was detected in the biosilica spheres. The remaining enzyme activity was detected in the supernatant, which indicated negligible loss of enzyme activity during immobilization. The immobilized enzyme showed excellent long-term and thermal stability.

Another approach to entrapment is the immobilization of the enzymes in hollow spheres^{264,265} or hollow fibers.²⁶⁶⁻²⁶⁸ In essence, the enzymes are separated from the bulk solution by a semipermeable membranes through which the substrates and products can pass. The size and nature of the substrates that can be handled are dependent on the pore size of the membrane and whether it is hydrophilic, hydrophobic or charged. A bioreactor of microcapsules would have the greater surface area through which diffusion can take place. However, with a system composed of bundled hollow fibers, a much greater quantity of enzyme can be used and the conditions within the fibers better controlled (pH, cofactors, trace metals, removal of products, etc.). In some instances the enzymes have been immobilized to or incorporated into the membranes themselves.²⁶⁹ In recent years, the primary emphasis on microencapsulated materials has been in therapeutic use, where the microcapsules or liposomes are intended to gradually break down in the body releasing their products (antibiotics, drugs, hormones, etc.). These microcapsules used in this application have generally been composed of polylactic acid (PLA) or polylactic-glycolic acid (PLGA) copolymer.

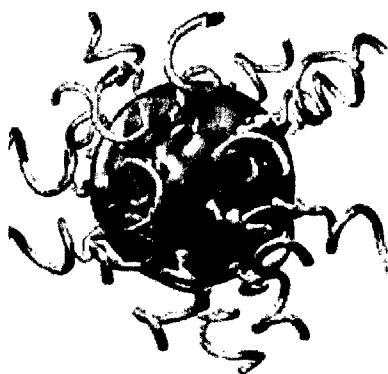
In the case of liposomes, lipid micelles containing either hydrophilic or hydrophobic contents, they were used to demonstrate the applicability of using organophosphorus-degrading enzymes therapeutically or prophylactically. In two studies conducted by James Wway, Texas A&M University, OPAA and OPH were encapsulated in sterically stabilized liposomes and injected into mice. The liposomes were stable in blood for over two days while maintaining full enzymatic activity. The standard antidote for organophosphorus poisoning is atropine and pralidoxime (2-PAM). These materials alone could protect the mice against ~5 LD₅₀'s of DFP. The OPAA-liposomes could also protect against ~5 LD₅₀'s of DFP. However, when the liposomes were combined with atropine and 2-PAM, the mice were protected against ~25 LD₅₀'s of DFP.^{270,271} In the case of OPH-liposomes, the combined treatment protected mice against more than 1000 LD₅₀'s of paraoxon.²⁷² Liposomes can be stabilized by means of cross-linking, but generally are not as robust as the microcapsules made from synthetic polymers.

3.2 Stabilization Methods.

Enzymes such as amylases, cellulases, proteases, etc., that are secreted by cells and function in the extracellular environment often have high intrinsic stability.

This is especially the case for enzymes produced by extremophilic microorganisms. However, the vast majority of enzymes function within the protected environment of the cell interior. Therefore, they may or may not have the stability required for extended use in decontaminating/disinfecting water supply systems. This can only be determined by testing them in the environment in which they would be used. If they are not sufficiently stable, there are basically two methods available to deal with the situation. They can be chemically modified (cross-linked or attached to water-soluble polymers) or the enzyme itself can be modified through genetic engineering.

By far, the chemical modification that has been used the most is linkage to polyethylene glycol (PEG) a waxy, nontoxic polymer of varying chain length. Initially, this technology was developed to assist in the use of enzyme therapy. The difficulty in using enzymes therapeutically or prophylactically is that they are quickly cleared from the blood stream, sometimes in as little time as minutes. As shown in the cartoon below, the concept of pegylating enzymes was to cover them with the PEG polymers that do not have a permanent or defined structure. Thus, there is nothing for the immune system to develop antibodies against.



PEG modified bovine adenosine deaminase (ADA) was first used experimentally in 1987 to successfully treat two children with ADA deficiency.²⁷³⁻²⁷⁵ It received FDA approval in 1990 and is sold under the name ADAGEN® by Enzon, Inc., along with ONCASPAR® (1994, asparaginase for treatment of acute lymphoblastic leukemia) and PEG-INTRON® (2001, interferon as part of a treatment for Hepatitis C). In 1988, Hershfield's group also reported on their study of PEG-modified uricase to treat hyperuricemia.²⁷⁶ However, in addition to providing immunological invisibility, PEG can also confer enhanced stability to enzymes. PEG-modified oxalate oxidase was found to have increased storage stability, higher thermal stability, and resistance to heavy metal inactivation and proteolytic digestion²⁷⁷ and other proteins have yielded similar enhancements.²⁷⁸ PEG is an amphiphilic polymer that provides a water-like microenvironment for the enzyme, and this enables the modified enzyme to work in hydrophobic macroenvironments such as organic solvent.²⁷⁹ Other methods that have shown some enhancement of enzyme stability include attachment carbohydrate chains, reaction with glyoxylic acid, and acetylation.

Another method that has been used is to modify the enzyme by cross-linking intramolecular groups. Some of the materials used include bifunctional PEG's, bifunctional imidoesters (dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate), and modified succinimides.²⁸⁰⁻²⁸⁶ For each enzyme, it is a matter of trial and error to determine which modification will result in the most stable, as well as active, product.

The alternative to chemical modification is to change the structure of the enzyme protein itself. Of course, this requires that the gene(s) that code for the enzyme are cloned, sequenced and expressed. If the three-dimensional structure of the enzyme has been determined, specific changes in selected amino acid residues can be made (site-directed mutagenesis). These could include replacing heat labile asparagines with glutamines, increasing salt bridges, disulfide bonds or hydrophobic interactions (aimed at making protein unfolding more difficult), and enhancing the binding of metals or prosthetic groups.²⁸⁷ With enhancements in molecular modeling and molecular biology, this would seem to be relatively straightforward. However, the structure of the enzyme may be very difficult to obtain, and the crystal structure may have significant differences to the solution structure. While advancements in NMR technology are improving the potential of getting highly accurate protein solution structures, there are still limitations on the degree of resolution and the size of the protein.

One way to get around the problems of site-directed mutagenesis is to generate random mutations throughout the protein. The library of mutants is then screened under conditions where those with the desired trait can be detected (enhanced temperature stability, change in pH optimum, altered substrate specificity, resistance to inhibitors, etc.). There is a variety of methods to generate these random mutations. Some, generically called random mutagenesis or DNA shuffling,^{288,289} involve the use of error-prone PCR and other techniques to introduce errors into the gene *in vitro*. The mixture of modified DNA's is then cloned back into a host strain for screening. Another method creates the mutations *in vivo*. This makes use of a strain of *E. coli* that has a mutation rate about 5000-times higher than normal. The enzyme gene is cloned into this strain and the bacteria are allowed to grow for varying periods of time. The DNA is then extracted and cloned back into a normal host strain for screening. With either method, when enhanced mutants are identified, they can be subjected to a second or third or more generations of mutation. This method has worked quite well at ECBC in which a bacterial urease enzyme has been run through several generations to yield greatly increase its resistance to fluoride inhibition²⁹⁰ and oxidation by hydrogen peroxide.

The most challenging part of these methods is the screening, both in selecting the most appropriate parameters and the sheer numbers. The various techniques for generating mutations can result in 5,000 to 1,000,000 potential mutants that require screening from a single experiment. While this can sometimes be done on the agar plates onto which the mutant library is plated, it is often not the case so that assays in liquid need to be carried out. This is most efficiently done through the use of robotic high throughput screening (HTS) systems that can pick colonies from agar

plates, prepare and initiate reactions in 96-, 384- or 1152-well plates, monitor the reactions, and collect the results. Where a person could potentially conduct ten's or perhaps hundred's of screens an eight-hour day, an HTS system can easily do ten's of thousands. In addition, it is feasible to have the robotic system running 24 hours a day. Various types of HTS systems are being used extensively in the pharmaceutical and biotechnology companies. However, the current cost (\$200-300,000) of the systems limits their availability in many other laboratories. At the moment, ECBC does not have such a system that could be used on this project.

4. CONCLUSIONS

It appears that the types of chemical or biological agents that could pose a threat to water supply systems can be dealt with using catalytic enzymes. However, it is also apparent that different agents or operations may well require different approaches. For example, in dealing with chemically contaminated pipes, it would probably be advisable to also plan on dealing with any biofilm that may be present since it could absorb and shelter the agent. Whether this is accomplished with a single formulation at one time or sequentially (biofilm destroyers first, followed by chemical agent enzymes) will depend on the compatibility of the various enzyme components. If the chemical agent enzymes are modified with PEG, they may be protected from the proteases, etc., that will be necessary to deal with biofilms. The destruction of biological agents in pipes would primarily be an upgrading of the biofilm destroying system, if necessary, to deal with a particular organism or toxin.

For dealing with flowing water, enzyme filters can certainly be used for the chemical agents and toxins. This assumes that there are no significant diffusion limitations. For maximum stability, activity and throughput, either hollow-fiber or encapsulated/entrapped enzymes would appear to be the best formats to employ. For biological agents the use of enzyme filters is more problematic because of the size of the target and the contact issue. However, there has been a considerable amount of research done recently on chemically modifying surfaces to make them bactericidal.²⁹¹⁻²⁹³ If the hollow fibers or enzyme capsules were coated with these materials, or coated particles were included in the system they may provide significant killing of biological threat organisms as well as inhibiting the development of biofilms on the surfaces of the filter.

For the laboratory phase of this project, evaluating the various immobilization and stabilization methods can be carried out with enzymes either already at ECBC (OPH, OPAA and possibly a dehalogenase) or available commercially. Precisely which substrates to be used in the validation portion of the project will be agreed upon in advance with EPA.

A potential threat to water supply systems that was not discussed in this review was from toxic metals or radioactive materials. While neither can be "decontaminated/detoxified" by enzymes or any other method, the enzyme systems that

deal with biofilms could prevent them from remaining trapped in that type of reservoir. In addition, there are a variety of natural and synthetic chelators that can be used to remove these materials from water supplies.

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